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Materials for Fluorescence Resonance Energy Transfer Analysis: Beyond Traditional Donor–Acceptor Combinations**

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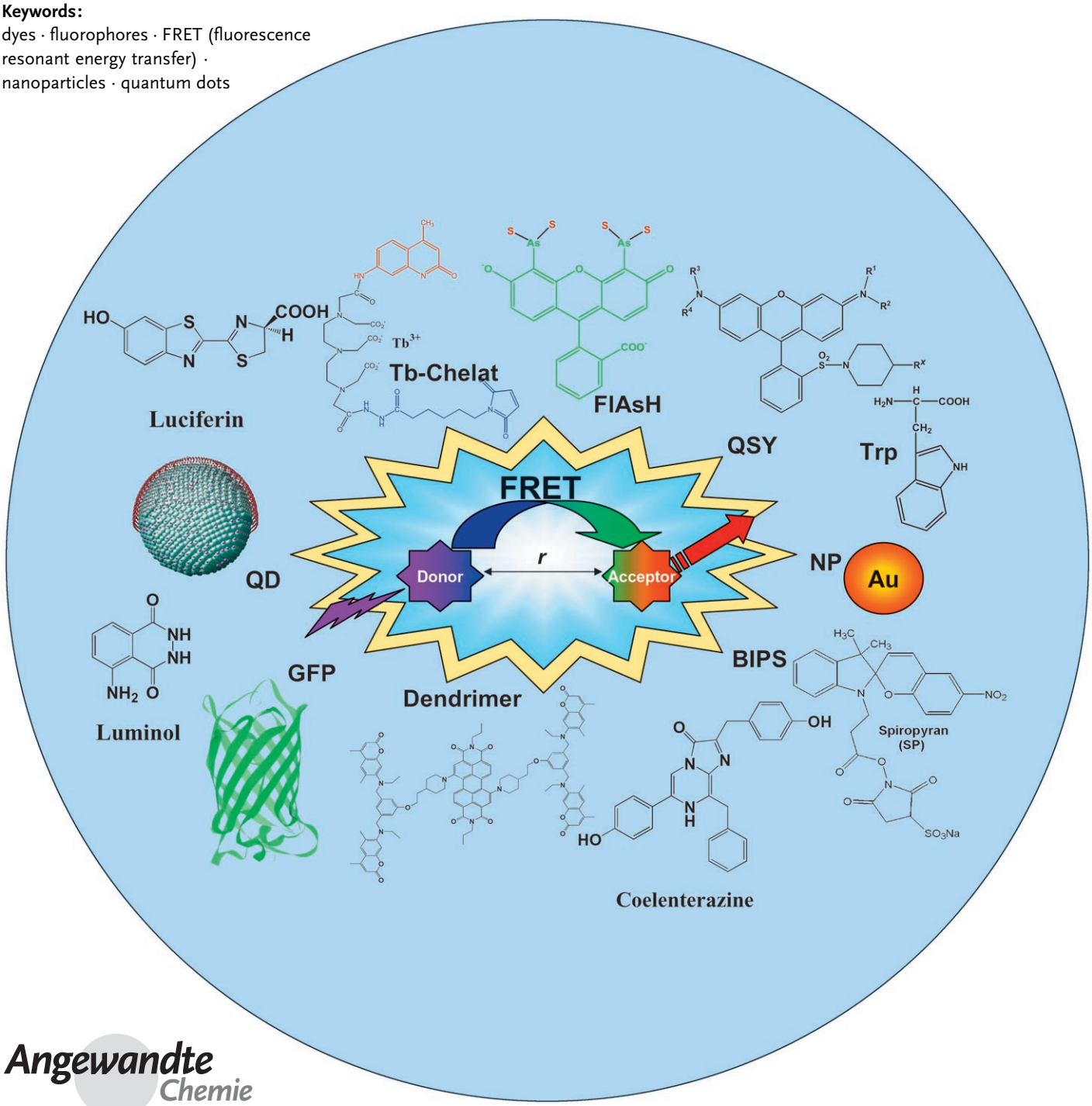

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The use of Förster or fluorescence resonance energy transfer (FRET) as a spectroscopic technique has been in practice for over 50 years. A search of ISI Web of Science with just the acronym "FRET" returns more than 2300 citations from various areas such as structural elucidation of biological molecules and their interactions, in vitro assays, in vivo monitoring in cellular research, nucleic acid analysis, signal transduction, light harvesting and metallic nanomaterials. The advent of new classes of fluorophores including nanocrystals, nanoparticles, polymers, and genetically encoded proteins, in conjunction with ever more sophisticated equipment, has been vital in this development. This review gives a critical overview of the major classes of fluorophore materials that may act as donor, acceptor, or both in a FRET configuration. We focus in particular on the benefits and limitations of these materials and their combinations, as well as the available methods of bioconjugation.

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1. Introduction

1.1. The FRET Process

Fluorescence resonance energy transfer (FRET) is a nonradiative process whereby an excited state donor D (usually a fluorophore) transfers energy to a proximal ground state acceptor A through long-range dipole–dipole interactions (Figure 1).[1,2] The acceptor must absorb energy at the emission wavelength(s) of the donor, but does not necessarily have to remit the energy fluorescently itself (i.e. dark quenching). The rate of energy transfer is highly dependent on many factors, such as the extent of spectral overlap, the relative orientation of the transition dipoles, and, most importantly, the distance between the donor and acceptor molecules.[2] An intensive description of the physical basis of FRET, which is beyond the scope of this review, can be found in reference [2].

FRET usually occurs over distances comparable to the dimensions of most biological macromolecules, that is, about 10 to 100 Å. Although configurations in which multiple donors and acceptors interact are increasingly common (see, for example, references [3–5]), the following equations consider energy transfer between a single linked D/A pair separated by a fixed distance r and originate from the theoretical treatment of Förster.[2,6,7] The energy transfer rate $k_T(r)$ between a single D/A pair is dependent on the distance r between D and A and can be expressed in terms of the Förster distance R_0. R_0 is the distance between D and A at which 50% of the excited D molecules decay by energy transfer, while the other half decay through other radiative or nonradiative channels. R_0 can be calculated from the spectral properties of the D and A species [Eq. (1)].

$$R_0 = 9.78 \times 10^3 [\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \text{ (in Å)} \quad (1)$$

The factor κ^2 describes the D/A transition dipole orientation and can range in value from 0 (perpendicular) to 4 (collinear/parallel). There has been much debate about which dipole orientation value to assign for particular FRET formats. Only in few cases can the crystal structure of the D/A molecules be determined; there is no other reliable experimental method to measure absolute or fixed κ^2 values, which leads to potential uncertainties in subsequent calculations.[2,8,9] Fortunately, the accumulated evidence has shown that the mobility and statistical dynamics of the dye linker lead to a κ^2 value of approximately 2/3 in almost all biological formats. This also sets an upper error limit of 35% on any calculated distance.[2] Excellent discussions of this issue are provided by dos Remedios and Moens[9] as well as Stryer.[10] The refractive index n of the medium is ascribed a value of 1.4 for biomolecules in aqueous solution. Q_D is the quantum yield (QY) of the donor in the absence of the acceptor and $J(\lambda)$ is the overlap integral, which represents the degree of spectral overlap between the donor emission and the acceptor absorption. The values for $J(\lambda)$ and R_0 increase with higher acceptor extinction coefficients and greater overlap between the donor emission spectrum and the acceptor absorption spectrum. Whether FRET will be effective at a particular distance r can be estimated by the "rule of thumb" $R_0 \pm 50\%$ R_0 for the upper and lower limits of the

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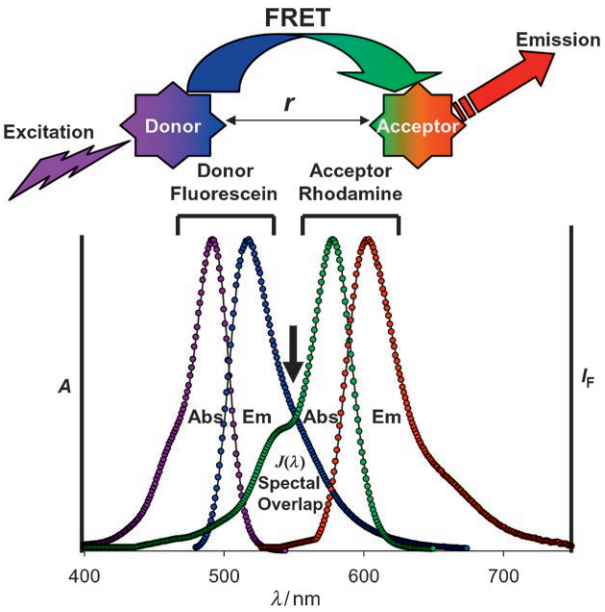


Figure 1. Schematic of the FRET process: Upon excitation, the excited-state donor molecule transfers energy nonradiatively to a proximal acceptor molecule located at distance *r* from the donor. The acceptor releases the energy either through fluorescence or nonradiative channels. The spectra show the absorption (Abs) and emission (Em) profiles of one of the most commonly used FRET pairs: fluorescein as donor and rhodamine as acceptor.[309] Fluorescein can be efficiently excited at 480 nm and emits at around 520 nm. The spectral overlap between fluorescein emission and rhodamine absorption, as defined by $J(\lambda)$, is observed at 500–600 nm. The Förster distance R_0 for this pair is 55 Å. Thus, in an optimal configuration ($r < 55$ Å), excitation of fluorescein at under 500 nm can result in significant FRET emission of the rhodamine at above 600 nm. A = normalized absorption, I_F = normalized fluorescence.

Förster distance.[8,9] The efficiency of the energy transfer can be determined from either steady-state [Eq. (2)] or time-resolved [Eq. (3)] measurements.

$$E = 1 - \frac{F_{DA}}{F_D} \quad (2)$$

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (3)$$

F is the relative donor fluorescence intensity in the absence (F_D) and presence (F_{DA}) of the acceptor, and τ is the fluorescent lifetime of the donor in the absence (τ_D) and presence (τ_{DA}) of the acceptor.

FRET is very appealing for bioanalysis because of its intrinsic sensitivity to nanoscale changes in D/A separation distance (proportional to r^6). This property is exploited in FRET techniques ranging from the assay of interactions of an antigen with an antibody in vitro to the real-time imaging of protein folding in vivo.[11,12] The myriad FRET configurations and techniques currently in use are covered in many reviews.[8,13,14] Herein, we focus primarily on the fluorophore materials utilized in bioanalytical FRET rather than the process itself. The materials can be divided into various classes: organic materials, which includes "traditional" dye fluorophores, dark quenchers, and polymers; inorganic materials such as metal chelates, and metal and semiconductor nanocrystals; fluorophores of biological origin such as fluorescent proteins and amino acids; and biological compounds that exhibit bioluminescence upon enzymatic catalysis. These materials may function as either FRET donors, FRET acceptors, or both, depending upon experimental design. A major focus is on FRET between disparate classes of materials; selected examples will be discussed for this purpose. We also focus on potential FRET materials that have not yet found practical application. Given the myriad examples available, we cannot do justice to all developments and we extend our apologies for any omissions.

1.2. Methods of Conjugating Fluorophores to Biomolecules

Fluorophore conjugation to biomolecules at known distinct locations is the most desirable FRET configuration; thus techniques for accomplishing this deserve some discussion. The most commonly used reagents for site-specific biolabeling are commercially available fluorophores with a succinimidyl ester or maleimide reactive group that targets the primary amino or thiol groups, respectively, on biomolecules such as proteins or DNA. As proteins have many primary amino groups (mostly lysine residues), the coupling is relatively unspecific and variable dye-to-protein (D/P) ratios result. Targeting thiol groups on cysteine residues with maleimide chemistry is more specific as these can be easily

Igor L. Medintz, born 1968, studied chemistry and forensic science at John Jay College of Criminal Justice, City University of New York. In 1998, he received his PhD in molecular biology under Prof. Corinne Michels of Queens College (also CUNY). He carried out postdoctoral research under Prof. Richard A. Mathies (UC Berkeley) on the development of FRET-based assays for microfabricated devices for genetic analysis. Since 2002 he has been at the Center for Bio/Molecular Science and Engineering of the US Naval Research Laboratory where he is working in collaboration with Dr. Hedi Mattoussi on creating biosensors with quantum dots.

Kim E. Sapsford, born 1974, studied chemistry at the University of East Anglia (Norwich, UK) and in 2001 received her PhD in analytical chemistry under Prof. David A. Russell. Since 2001 she has been at the Center for Bio/Molecular Science and Engineering of the US Naval Research Laboratory, where she is working on creating fluorescent-based biosensors using the array biosensor technology that was developed by Dr. Frances Ligler.

introduced into proteins recombinantly for this purpose.[15] However, this too can be problematic, since disulfide bridges already present in proteins may be critical to the conformation, and additional cysteine residues could destroy the protein structure. Additionally, proteins expressing even a single surface-exposed thiol group will form dimers when purified, so that a reduction step is necessary prior to labeling. The original chemistry for protein labeling was developed by Gregorio Weber, and many of the probes he developed are still in use today.[2] In general DNA and RNA labeling is less challenging, as these can be synthesized with site-specific thiol or amine groups, as well as nucleotides modified with a variety of fluorophores and quenchers.[16,17] Thus, both differential labeling and the exact placement of fluorophores within the oligonucleotide structure are possible. Table 1 lists some of the commonly available reactive groups on fluorophores designed for labeling biomolecules, along with their targets.

A variety of protocols exist for introducing specific functional groups onto biomolecules. Perhaps the best available resource on this subject is Hermanson's *Bioconjugate Techniques*.[18] *A Guide to Fluorescent Probes and Labeling Technologies* by Haugland is another good source (available free of charge from Molecular Probes).[19] Strategies exist that employ noncovalent or electrostatic interactions for associating fluorophores with biomolecules, although these are not so attractive for FRET applications.[2,19]

Several emerging technologies offer alternatives for site-specific fluorescent labeling of proteins; most are geared towards applications in vivo. Fluorescent proteins (FPs) such as the green fluorescent protein (GFP) can be appended onto existing proteins by using recombinant techniques, thus allowing the endogenous expression of fluorescent protein chimeras (see Section 3.2).[14,20,21] The FlAsH method (FlAsH = 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein) developed by Tsien allows in vivo coupling of nonfluorescent, cell-permeable biarsenical fluorophores to proteins expressing an optimized Cys-Cys-Pro-Gly-Cys-Cys sequence. Only the reacted fluorophore is emissive. This labeling technique has already been used for in vivo FRET applications (Figure 2).[21–24]

The HaloTag method utilizes a fusion protein with a dehalogenase domain, on which a fluorescent ligand is conjugated through substitution of a chloride function.[25] Another fusion protein based system, which allows both in vivo and solution labeling of target proteins, is the SNAP tag. This system utilizes a modified alkylguanine–DNA alkyl transferase, which reacts with a *p*-benzylguanine-modified fluorophore to form a thioether bond. Hellinga and co-workers have also described a method for the sequential/orthogonal labeling of multiple thiol groups on purified proteins by exploiting metal coordination and disulfide bond formation to protect cysteine residues in a Cys_2His_2 zinc-finger domain.[26] Future strategies may include in vivo incorporation of unnatural amino acids as unique labeling sites.[27] Regardless of the FRET method chosen, having both the donor and acceptor at known, distinct locations on biomolecule(s) is most desirable for analysis of the experimental data. It is also the most technically challenging to accomplish on a single molecular entity.

Table 1: Common reactive groups and methods for attaching fluorophores to biomolecules.[18,19]

Target	Reactive Group	Comment
thiol	maleimide, iodoacetyl, piridyldisulfide[a]	site-specific but requires a free cysteine on proteins
primary amine	succinimidyl esters (NHS), sulfonyl chlorides, iso(thio)cyanates, carbonyl azides[a]	proteins may have many primary amines
carboxyl	carbonyldiimidazoles, carbodiimides[b]	allows further coupling to amines
hydroxyl	carbonyldiimidazoles, periodate, disuccinimidyl carbonate[b]	allows further coupling to amines
carbohydrates	periodate[b]	oxidizes sugars to create reactive aldehydes, which couple to amines
intracellular proteins	FlAsH[22]	requires cloning
intracellular proteins	SNAP-tag/HaloTag[25]	requires cloning and commercial ligands
intracellular proteins	fluorescent proteins[14,20,21]	requires cloning and formation of a chimera

[a] Reactivity can also target amine- or thiol-modified DNA. [b] Multistep modifications.

Lorenzo Berti, born 1971, studied Chemistry at the University of Bologna (Italy) and obtained his PhD in organic chemistry from the same university in 2000. He carried out postdoctoral research under Prof. Richard A. Mathies at UC Berkeley, where he developed universal FRET tags for DNA sequencing and genotyping. Since 2003 he has been a Research Scientist at the INFM-CNR in Modena, Italy. His research interests include developing linking strategies for conjugating biomolecules to inorganic materials and exploiting the potential of DNA for engineering nanostructures.

2. Organic Materials

2.1. UV-, Vis-, and IR-Emitting Dyes

Organic dyes that emit in the ultraviolet (UV), visible (Vis), and near-infrared (IR) region are considered "traditional" FRET dyes. They represent the majority of D/A pairs currently used in FRET applications and are also the first type of dye usually tested with new or "nontraditional" materials. The most common are several structurally related classes of dyes whose emissions span the UV-to-IR spectrum (Figure 3). Such dyes are available in reactive form from commercial sources activated with *N*-hydroxysuccinimide (NHS) ester,

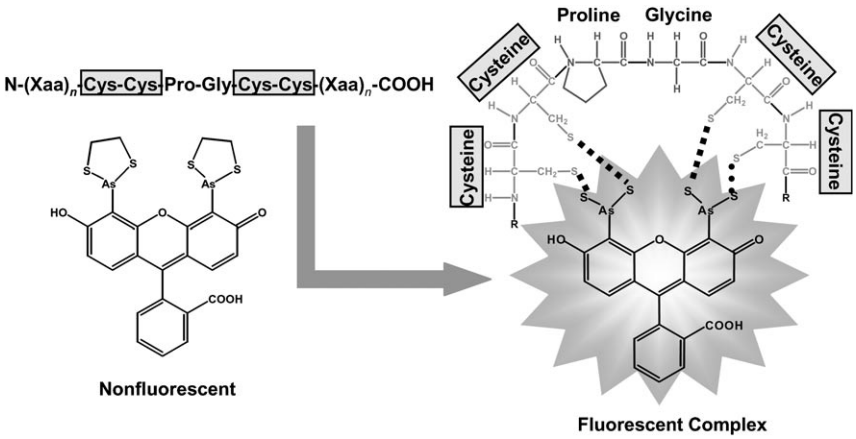


Figure 2. Structure of the fluorescein derivative FlAsH in its nonfluorescent form and the optimized hairpin motif with target cysteine residues highlighted (Xaa = generic amino acid). After conjugating with this peptide sequence in vivo, the bound fluorophore becomes emissive.[22,23]

Members of some dye families, such as the cyanines (Cy), are closely related in structure, whereas others, such as the AlexaFluor compounds, are quite diverse. All dye families are typically characterized by closely spaced, broad absorption/emission profiles (small Stokes shift) and all have both associated advantages and disadvantages depending on the intended application. For example, fluorescein dyes are popular because of their high quantum yields, solubility, and ease of bioconjugation, and fluorescence is readily obtained by excitation with a standard argon-ion laser (488 nm). However, fluorescein has a high rate of photobleaching, is pH-sensitive (which is sometimes advantageous, see Section 2.3), and can self-quench at high degrees of substitution. Alternatives such as Oregon Green dyes (fluorinated fluorescein analogues), the AlexaFluor compounds, the Cy family, and the

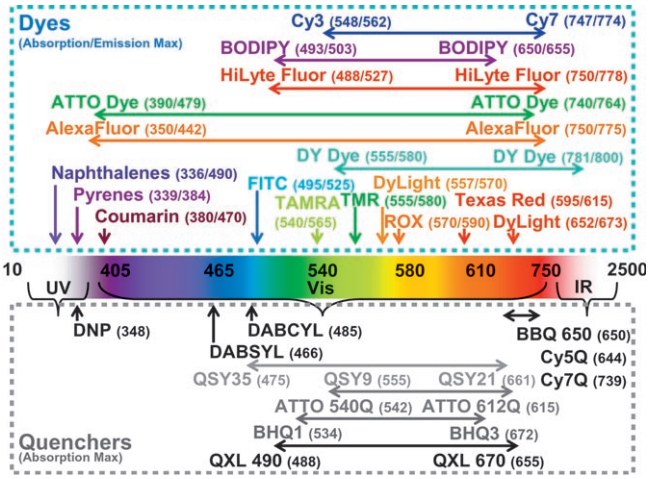
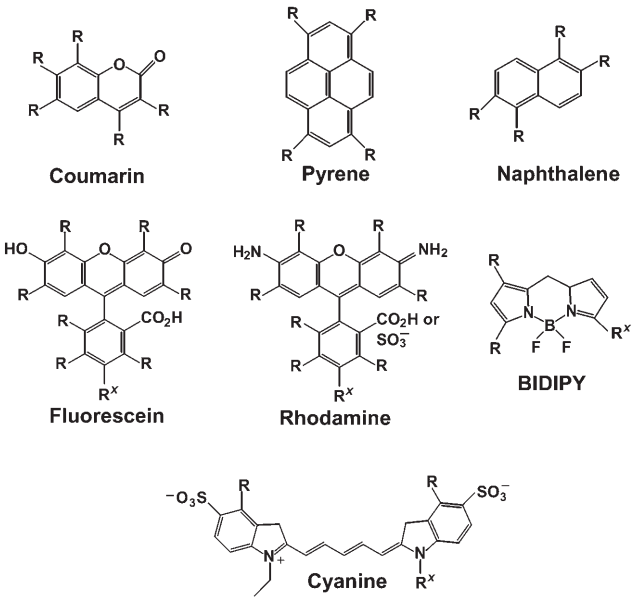


Figure 3. Examples of available fluorescent dye and quencher families, almost all of which have been used for FRET measurements. Absorbance and emission maxima along with spectral regions covered by a particular dye family are highlighted. Tetramethylrhodamine (TMR), carboxytetramethylrhodamine (TAMRA), and carboxy-X-rhodamine (ROX) are all rhodamine-based dyes. The most common D/A dye combinations are coumarin/fluorescein, fluorescein/rhodamine, and Cy3.5/Cy5. Popular dye/quencher combinations include rhodamine/Dabcyl and Cy3/QSY9. Major suppliers are the companies Molecular Probes (fluorescein, rhodamine, AlexaFluor, BODIPY Oregon Green, Texas Red, and QSY quenchers), Amersham Biosciences (Cy dyes and Cy5Q/Cy7Q quenchers), AnaSpec (HiLyte Fluors, QXL quenchers), ATTO-TEC (ATTO dyes and quenchers), and Molecular Biotechnology (DY dyes), Pierce (DyLight 547 and DyLight 647 dyes), Berry and Associates (BlackBerry), and Biosearch Technologies (Black Hole). FITC = fluorescein isothiocyanate.



Scheme 1. Structures of common UV/Vis fluorescent dyes. Typical substituents at the R position include CO_2^-, SO_3^-, OH, OCH_3, CH_3, and NO_2; R^x marks the typical position of the bioconjugation linker.

maleimide, hydrazide, or amine functionalities for bioconjugation. The UV dyes are typically pyrene-, naphthalene-, and coumarin-based structures, while the Vis/near-IR dyes include a variety of fluorescein-, rhodamine-, and cyanine-based derivatives (Scheme 1).

BODIPY compounds may alleviate some of these issues. Low solubility in aqueous environments can be an issue for some of the redder dyes, for which overlabeling can induce protein precipitation. For FRET applications in particular, the broad absorption/emission profiles and small Stokes shifts often lead to direct excitation of the acceptor, which complicates subsequent analysis. In general, their advantages include: the availability from commercial sources, their relatively low cost, the availability of established methods for bioconjugation, and, most importantly, the extensive description of their FRET properties in the literature.

There are many resources available to aid in choosing suitable D/A pairs, including a number of reviews.[2,15,28,29] Wu and Brand[30] offer an extensive list of donor–acceptor dye pairs and their respective R_0 values. Haugland's *Handbook* is another excellent resource.[19] The web-based programs of Invitrogen (http://probes.invitrogen.com/resources/spectraviewer/) and BioRad (http://microscopy.biorad.com/fluorescence/fluorophoreDatab.htm) allow the researcher to plot multiple dye absorption/emission profiles to optimize spectral overlap as well as choose appropriate filters. Buschmann et al. also give an excellent comparison of the physical and spectroscopic properties of a number of red-absorbing dyes.[31]

Dye-to-dye FRET combinations still remain state-of-the-art for many applications.[2,8] Enzymes, designed substrates, and cell surface receptors labeled with these organic D/A dye pairs have been used both in vitro and in vivo to monitor various biochemical processes, such as 3′,5′ cyclic monophosphate (cAMP) production,[32] phosphodiesterase activity,[33] β-lactamase activity,[34] integrin binding,[35,36] as well as conformational and electrical processes in single- ion channels.[37] Similar dye combinations are also useful for FRET-based biosensors, for examples, hydrogel-encapsulated glucose sensors[38] and sensors for lysozyme,[39] zinc,[40] and cholera toxin.[41]

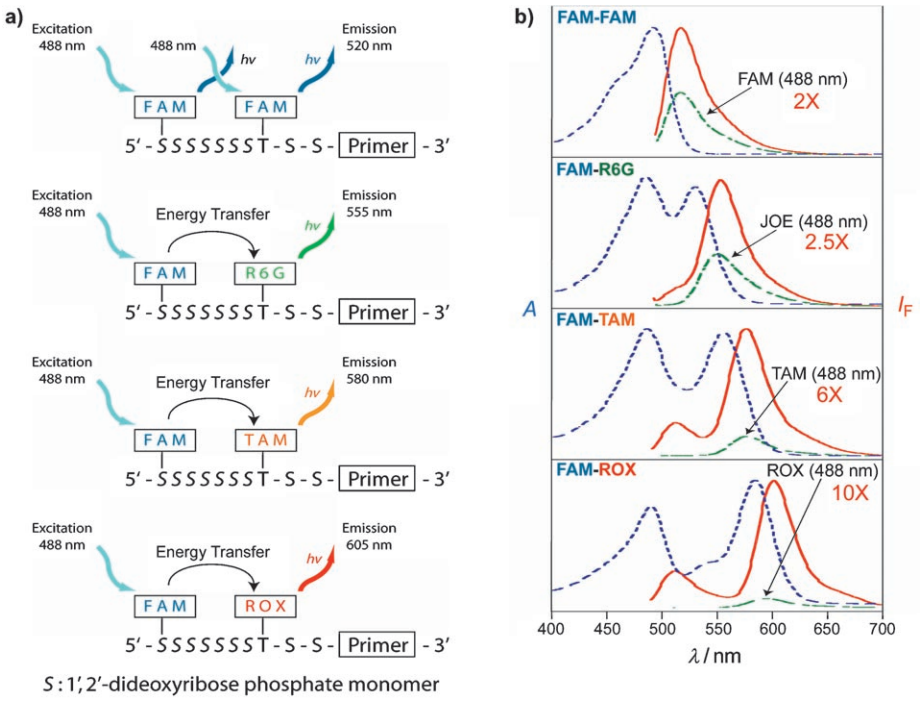


Figure 4. a) Schematic of energy-transfer cassette primers.[17] Each of the cassettes is built upon a common modified sugar-nucleotide backbone. The use of different acceptors creates four spectrally separated emission windows centered at 520, 550, 580, and 605 nm. The energy-transfer cassettes are attached to thiolated primers through thiol bridges. R6G = 6-carboxyrhodamine-6-G, TAM = carboxytetramethylrhodamine. b) Normalized absorbance and emission spectra (488-nm excitation) for each of the four colors of an ET cassette compared with direct excitation of the corresponding control acceptor dye at the same concentration. The factor by which the emission is increased in the FRET system relative to the single dye control assay is indicated in red. Figure generously provided by R. Mathies, UC Berkeley.

One area in which FRET applications with donor–acceptor dye combinations has had tremendous impact is nucleic acid analysis, particularly DNA sequencing and genotyping.[16,28,29,42] Mathies, Glazer, and co-workers realized that the use of a FRET system could simplify the instrumentation needed for DNA sequencing. By utilizing a common donor and four different acceptors (one for each of the DNA bases) attached to common DNA primers, they created four well-separated spectral emission windows that were excited at only a single wavelength.[43–45] The use of FRET could increase the acceptor emission with these primers by over 20 times with respect to directly excited non-FRET controls. This FRET strategy became the backbone of the DNA analysis technology that has revolutionized genomics and is found in derivative genotyping technology such as the Taqman assay.[16] A cassette version of the ET primers was created for attachment to any desired thiolated primer or oligonucleotide (Figure 4).[17] FRET-based DNA sensors have also been used to monitor pH variations in living cells during apoptosis.[46]

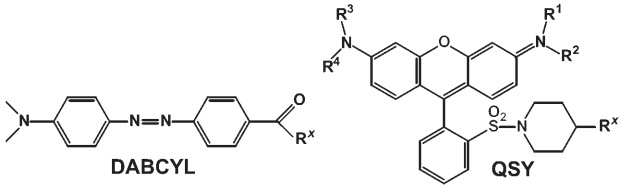
Interestingly, the use of DNA scaffolds has helped address fundamental questions about the dependence of the FRET efficiency on the orientation of the D/A dyes.[47] The immobilization of FRET-based DNA probes onto glass[48] and gold[49] has recently been tested and will be important for utilizing such probes in high-throughput parallel detectors (analogues of DNA microarrays).

It is clear that traditional dye-to-dye FRET systems will continue to play an important role. The substitution of the donor and acceptor with other classes of fluorophore will be driven by addressing deficiencies of organic dyes and creating new applications.

2.2. Quencher Molecules

The use of quenching acceptors is becoming increasingly popular in FRET systems. The principal advantage that these molecules offer over their fluorescent counterparts is the elimination of background fluorescence originating from direct acceptor excitation or re-emission.

Quenchers can take the form of organic molecules or metallic materials such as gold (Section 4.2). There are a variety of organic quencher families available commercially (Figure 3 and Scheme 2). Dabcyl (4-(4′-dimethylaminophenylazo)benzoic acid) and Dabsyl (4-dimethylaminoazobenzene-4′-sulfonyl) are two of the most common nonfluorescent acceptors, with absorption maxima centered at 485 and 466 nm, respectively. Other quencher families include the QSY, QXL, ATTO, BlackBerry, and Black Hole quenchers.



BHQ	R^1	R^2	R^3	R^4	R^5	R^6
1	CH_3	NO_2	CH_3	H	H	OCH_3
2	NO_2	H	H	OCH_3	OCH_3	H
10	NaO_3S	H	H	NaO_3S	H	H

Scheme 2. Structures of common organic quencher molecules. The substituents R are listed; R^x marks the typical position of the bioconjugation linker.

These generally tend to have broad absorption spectra, which allow them to function as acceptors for many dyes. Quenchers are often applied to DNA analysis, in particular, in molecular beacons in the form of acceptors paired with organic dye donors (see review articles by Tan et al.[28,29] and Didenko[16]). The principal advantage of this configuration is that it allows monitoring of the donor channel alone and if sufficient spectral separation is achieved opens the possibility of "multiplexing" with other donor/quencher pairs. Besides DNA-based diagnostics, molecular beacons incorporating quencher species have been used to measure DNA permeability of polyelectrolyte thin films,[50] and catalytic DNA biosensors have detected lead ions.[51] Quencher-labeled substrate analogues have been used in conjunction with dye-labeled proteins for FRET-based displacement biosensing of nutrients.[52] One of the few examples of a FRET system in which organic quencher molecules are coupled to a non-organic fluorophore involves quantum dot (QD) donors (see Section 4.3).

2.3. Environmentally Sensitive Fluorophores

Environmentally sensitive fluorophores exhibit some change in their absorbance and emission properties in response to a change in their environment such as pH, ionic strength or type, (e.g., Ca^{2+}, Cl^-), O_2 saturation, solvation, or polarity. It is difficult to define them as a completely discrete class of fluorophores, as almost all fluorophores respond to some perturbation in their environment.[2,19] Thus, these fluorophores are usually defined by the analyte or condition to which they respond most favorably (e.g., pH or calcium indicator dyes).[19] Perhaps the best known example is fluorescein (FAM; Scheme 1), whose absorption and emission change in response to pH as a function of ionization equilibria (Figure 5).[19] This property has been extensively exploited to monitor intracellular pH, and a variety of FAM ester derivatives are available and are retained intracellularly following delivery and ester hydrolysis. The most commonly

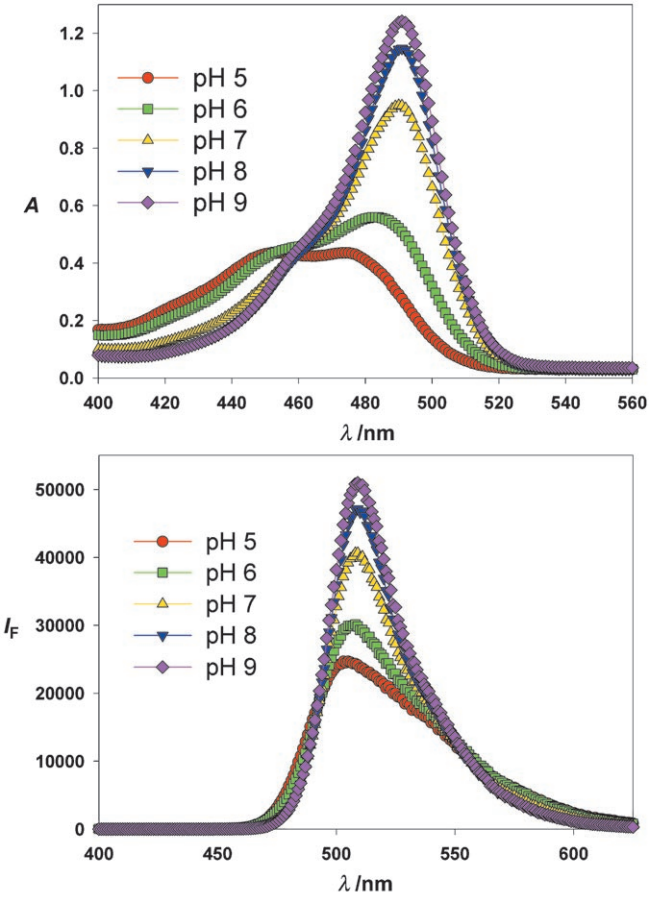


Figure 5. pH-dependent absorption (top) and emission (bottom) spectra of fluorescein. The largest change is between pH 6 and pH 7.

used probe for estimating intracellular pH is the polar BCECF derivative developed by Tsien and co-workers.[53] For pH monitoring, Molecular Probes offers a variety of reactive FAM analogues and proprietary seminaphthorhodafluors (SNARF), seminaphthofluoresceins (SNAFL), and their ester derivatives.[19] The optimal working range of these fluorophores is around pH 5–9. For acidic solutions, Oregon Green and Lysosensors are more appropriate.[19] However, the emission of these dyes is confined to the visible and near-IR region, and many are not available with reactive groups, so extensive chemical modification may be necessary to attach them to biomolecules or other dyes. Again, Haugland's *Handbook*[19] is a good reference for probes optimized for monitoring pH, NO_2, Ca^{2+}, Mg^{2+}, Zn^{2+}, Na^+, Cl^-, K^+, and membrane potential.

Several other dyes such as acrylodan and pyrene have also been used as biosensors for changes in the environment of the coupled protein.[15] Lakowicz and co-workers have utilized FRET with environmentally sensitive acceptors to measure pH values, as well as CO_2 and NH_3 concentrations by using phase modulation fluorometry,[54,55] a technique that requires specialized equipment and expertise. In general, FRET configurations with environmentally sensitive fluorophores have not been utilized extensively as most of the fluorophores function adequately alone. Future applications of FRET-

based environmental sensors could facilitate spatiotemporal-correlated multicolor measurements of intracellular conditions by working in conjunction with other dyes.

2.4. Dye-Labeled Microspheres/Nanoparticles

One of the limitations of conventional fluorescent assays is the difficulty in conjugating more than one fluorophore to a target. Conjugation to multiple fluorophores can increase the signal and achieve lower limits of detection; however, it can alter the function of the target biomolecule. To overcome these limitations, functionalized polymeric microspheres have been "soaked" with fluorophores, resulting in highly fluorescent nano- and microscale particles. Besides the increase in fluorescence intensity, fluorescent microspheres present other advantages. For instance, fluorophores that are water-insoluble or lack a reactive group can be loaded into microspheres. Fluorescent microspheres that absorb and emit from the UV to the near IR and whose sizes can range from 2 μm down to around 20 nm are available from, for example, Molecular Probes, Bangs Laboratories, and Polysciences. Molecular Probes also offer TransFluospheres, which are microspheres loaded with a proprietary combination of dyes that optimize internal FRET to yield large Stokes shifts.[56] Fluorescent microspheres are also provided with a variety of surface functionalities (e.g., biotin, avidin, collagen, amines, aldehydes, sulfates, and carboxylates) that allow facile bioconjugation to targets of interest. Functionalized spheres can also be purchased and soaked with dyes by following published procedures.[57,58]

Fluorescent microspheres have been extensively employed in FRET-based analytical assays, especially flow cytometry[59–61] and SNP genotyping.[62] Besides standard fluorophores, fluorescent microspheres loaded with Eu-based fluorophores are available which can be used for time-resolved measurements of the energy transfer (Section 4.1).[63] Apart from flow cytometry, the use of fluorescent microspheres for FRET-based assays is still in their infancy. However, their high fluorescence intensity, wide absorption with multiple emission colors, multifaceted chemistry, and commercial availability make them promising as donors, especially for multiplex FRET assays.

2.5. Dendrimers and Polymers

Dendrimers are highly-branched polymers that are obtained by stepwise synthesis.[64] A typical dendrimer contains a core monomer from which multiple branches stem. Each branch can be further expanded by adding other layers of monomers.[65] The principal utility in the current context is that multiple fluorophores and other chemical functionalities, which may be modified further, can be conjugated or adsorbed to the external shell to create highly fluorescent dendrimers.[66–71] Inherently fluorescent dendrimers have also been synthesized.[72,73]

The oriented placement of dyes can allow the energy absorbed at the periphery of the dendrimer to be funneled through intramolecular energy transfer to a common acceptor positioned at the core; thus the dendrimer effectively acts as an artificial light-harvesting antenna (Figure 6, top).[74–77] The

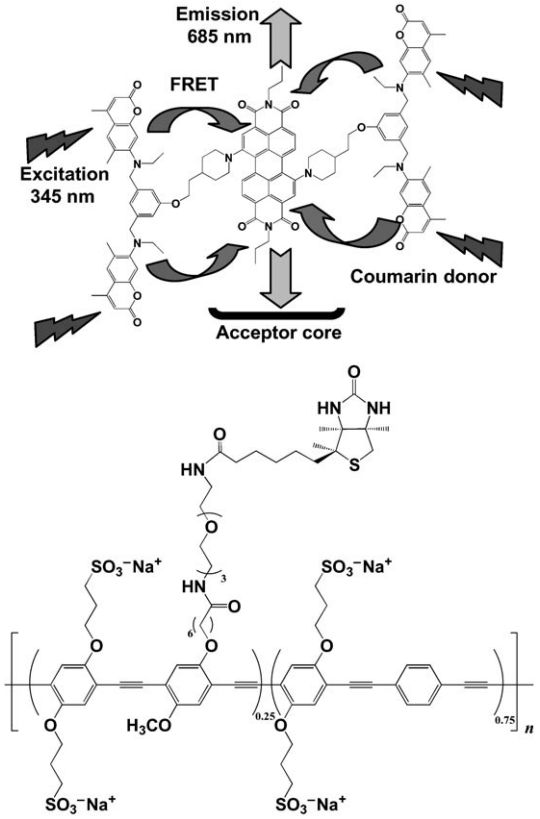


Figure 6. Top) Structure of a FRET dendrimer derived from a perylene bis(dicarboximide) acceptor as the core and a coumarin functionalized shell as the donor.[76] UV light (345 nm) is absorbed at the periphery, transfered as electronic energy to the acceptor in the core, and from there emited in the near-IR (685 nm; 99% ET efficiency). Bottom) Structure of a biotin–polymer conjugate, employed in the detection of DNA hybridization.[98]

major advantages of dendrimer-based fluorophores in bioassays are the increased absorption cross section and higher fluorescence intensity.[78,79] Furthermore, as the solubility is determined by the dendrimer, it is possible to deliver a drug or a fluorophore to an environment in which it would otherwise be insoluble.[80] Dendrimers have been used, for example, as carriers for a variety of labels including metal nanoparticles[81,82] and oligonucleotides,[83] as well as for in vitro probes and in drug-delivery assays.[84,85] Intermolecular energy transfer between dendrimers and other donors or acceptors are known but not common. Examples include FRET from dendrimers to pyrene polymers in Langmuir–Blodgett multilayers[86] and between dendrimers.[87]

Dendrimers with functionalities that can be further modified by the end user are commercially available (Dendritech and Dendritic Nanotechnologies). Genisphere commercializes DNA-based dendrimers that can be used in hybridization and detection of low-copy target genes. Qiagen

offers dendrimers functionalized to bind both DNA and cells for cellular transfection. Glen Research commercializes monomers for generating multibranched synthetic DNA dendrimers[88,89] that can be employed for labeling oligonucleotides with multiple fluorophores. Such multilabeled dendrimeric primers can also be employed for high-sensitivity, multiplex PCR analysis (PCR = polymerase chain reaction). The corresponding synthetic methods are also available.[64]

Fluorescent polymers are a related class of fluorophores that can be either intrinsically fluorescent (e.g., conjugated polymers) or functionalized with multiple fluorophores.[90–94] Similar to dendrimers, fluorescent polymers are characterized by high molar absorption coefficients and are effective light-harvesting antennas. Disadvantages for their use as fluorescent labels for bioconjugation are their size and polydispersity. The emission of fluorescent polymers is not localized, since energy transfer occurs along the whole chain and thus the emission is diffuse.[95] Polymers therefore cannot be used as point donors in FRET systems. Nonetheless, fluorescent polymers have found broad application as fluorescent layers and in thin films for biosensors.[96,97]

Polymer-based FRET systems have been successfully employed in developing highly sensitive bioassays by exploiting a phenomenon known as superquenching. Superquenching is a photophysical phenomenon whereby certain fluorescent polymers act as strong fluorescence quenchers when associated through electrostatic interactions with small molecules.[98] It occurs by a very efficient energy-transfer mechanism that is possible in solution and on surfaces. The effect has been used in DNA hybridization (Figure 6, bottom),[98] SNP analysis,[99] and protease detection.[100] Swager and co-workers have harnessed this phenomenon to develop "amplified fluorescent polymer sensors" for a variety of explosives and biological moieties.[97,101,102] Superquenching also occurs in conjugates of gold nanoparticles and fluorescent polymers;[103] such systems may be exploitable for bioassays.

2.6. Photochromic Dyes

Jovin and co-workers define photochromic compounds as "having the ability to undergo a reversible transformation—in response to illumination at appropriate wavelengths—between two different structural forms having different absorption (and in some cases, fluorescence) spectra".[104] The primary attraction of using photochromic dyes as FRET acceptors is the possibility of reversibly switching the acceptor (and hence the FRET effect) "on" and "off" with light. Many interesting FRET configurations can be constructed with this concept.

Spiropyrans and functionally related molecules are among the more prominent photochromic compounds. These molecules exist in closed spiro forms (absorbance < 400 nm) that undergo a light-driven intramolecular rearrangement to an open merocyanine form (absorbance 500–700 nm; Figure 7a).[105] Jovin and co-workers have synthesized a family of substituted diheteroarylethenes as photoswitchable accept-

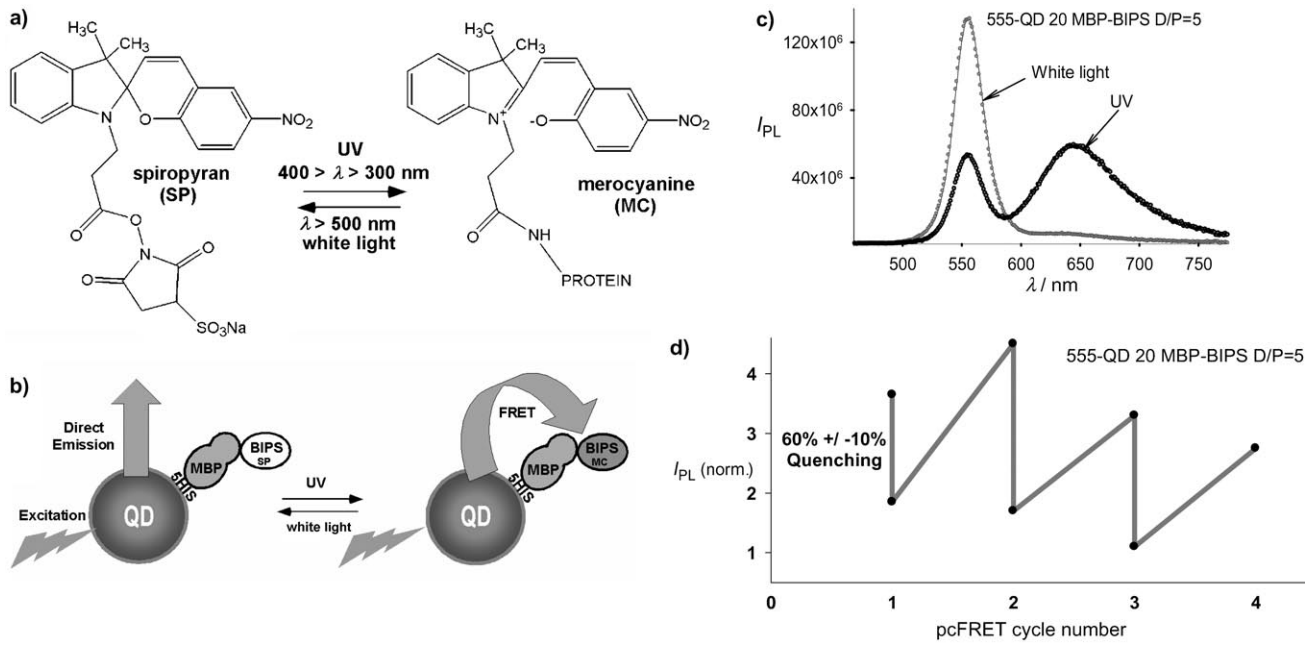


Figure 7. a) Structure of sulfo-NHS-BIPS (sulfo-NHS = *N*-hydroxysulfosuccinimide sodium salt; BIPS = 1′,3′,3′-trimethylspiro[2*H*-1-benzopyran-2,2′-indoline]) in the spiropyran (SP) form before (left) and merocyanine (MC) form after (right) conjugation to a protein. b) Schematic representation of quantum dot (QD) modulation by photochromic FRET after interacting with MBP-BIPS (MBP = maltose-binding protein). When BIPS is converted to the MC form by UV light, the QD emission is reduced through FRET quenching. After photoconversion with white light to the SP form, the direct emission of the QD is substantially increased. c) Photoluminescence spectra of the 555-nm luminescing QD 20 MBP-BIPS system with a dye/protein ratio of 5 after photoconversion from the SP to the MC form. d) Effect of pcFRET on QD photoluminescence (initial change from white light to UV). Figure adapted from reference [106] with permission of the American Chemical Society.

ors for what they term photochromic FRET (pcFRET). Using Luficer Yellow as a donor, they demonstrate 100% FRET efficiency in 40 consecutive pcFRET switching cycles without photophysical fatigue.[104] A further pcFRET system consisted of a nitrospiropyran acceptor linked to a porphyrin donor as a free base or complexed to zinc.[105]

A system consisting of a QD donor surrounded by multiple spiropyran acceptors has also been investigated for pcFRET (Figure 7b–d).[106] Altering the number of acceptors around the central QD donor modulated the pcFRET efficiency to between 25 and 50%. Other photochromic dyes include substituted perfluorocyclopentene, dithienylethenes, substituted oxazolylfulgides, and bismuth vanadate pigments.[107–109] A variety of spironaphthoxazines and naphthopyrans (known as Reversacols) are available in more than 20 different colors from the company James Robinson.

There are inherent benefits to pcFRET. As a result of the switching properties, two interconvertible FRET sensors with different photophysical characteristics can be obtain from a single configuration. The choice of switching wavelength can be such that it does not overlap with the absorbance of the donor. Jovin and co-workers have postulated that the use of pcFRET could overcome problems in quantitative cell-based FRET analysis (high local sensor densities, irreversible photobleaching with continuous monitoring).[104] Although the pcFRET process remains fascinating, a realistic biological system has not yet been demonstrated.

3. Biological Materials

3.1. Natural Fluorophores

There are many naturally occurring, intrinsically fluorescent biomolecules, including several enzymatic cofactors and the aromatic amino acids, tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), which are the focus of this review (Scheme 3).[2] Perhaps the single biggest benefit from using these residues for fluorescence is their endogenous presence in proteins/peptides and the ease with which they can either be introduced into proteins recombinantly or synthesized into nascent peptides. The strong UV absorbance of proteins at 280 nm (commonly used for quantitation) as well as the emission at 340–360 nm originate mostly from the indole ring of the tryptophan residue; tyrosine and phenylalanine contribute to a much lesser extent.[2] The negligible quantum yield (ca. 0.02) of phenylalanine makes it less amenable to FRET, except perhaps in intraprotein configurations. Tyrosine is prone to quenching and energy transfer to tryptophan. This leaves tryptophan as the most reliable residue for FRET (see review articles and references [2,30,110]). A potential drawback to FRET applications with Trp is that the excitation lines and any D/A dyes will be confined to the UV region. The fluorescence from these residues is also environmentally sensitive and so placement of these residues deep within a protein structure will produce results that differ from those at the terminus of a small peptide. As an example of protein fluorescence, the absorption and emission spectra of the maltose-binding protein (MBP) are shown in Figure 8. MBP is a well-characterized member of the bacterial periplasmic binding protein (bPBP) superfamily and contains 8 Trp, 15 Tyr, and 15 Phe residues.[15]

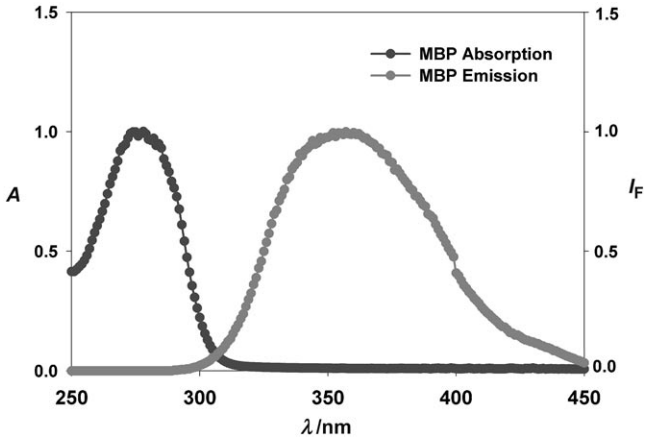
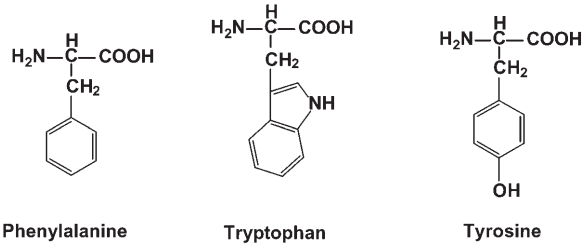


Figure 8. Normalized absorption and emission profile ($\lambda_{ex} \approx 280$ nm) of maltose-binding protein (MBP; $M_r \approx 44{,}000$).[4,15,129]



Scheme 3. Structures of the three naturally fluorescent aromatic amino acids phenylalanine (Phe: QY = 0.02, $\tau \approx 7$ ns, $\lambda_{ex} \approx 260$ nm, $\lambda_{em} \approx 282$ nm), tryptophan (Trp: QY = 0.13, $\tau \approx 3$ ns, $\lambda_{ex} \approx 295$ nm, $\lambda_{em} \approx 353$ nm), and tyrosine (Tyr: QY = 0.14, $\tau \approx 3$–4 ns, $\lambda_{ex} \approx 275$ nm, $\lambda_{em} \approx 304$ nm).[2,110]

Myriad examples highlighting the versatility of these endogenous fluorophores abound. These include a FRET system with a Trp donor and dansyl acceptor to estimate the helix–helix association of bacterioopsin.[111] A Trp donor has also been used to measure the binding affinities for the *E. coli* DEAD-Box RNA helicase DbpA with fluorescent nucleotide analogues as acceptor.[112] A Trp located within the core of a reductase protein functioning in conjunction with a NADPH coenzyme quencher was used for measuring binding affinities.[113] Trp residues within the *E. coli* melibose permease acting as energy donors for a fluorescent sugar analogue were identified through their sequential mutagenesis.[114] A FRET system consisting of a fixed Trp residue as donor and a modified 3-nitrotyrosine as acceptor within the human α-synuclein protein was used to demonstrate that the elongated structure of a mutant is associated with Parkinson's disease.[115] Trp has also been used as an acceptor for a nitrile-derivatized phenylalanine donor to study the conformation of a 14-residue amphipathic peptide.[116] The distance between helices in the M13 transmembrane procoat protein was

measured by FRET with a Tyr donor and a Trp acceptor.[117] Other FRET configurations include a Trp donor and a chromium(III) acceptor,[118] and a homotransfer system between Trp residues.[119]

Residue-to-residue FRET is more advantageous for smaller distances (< 5 nm) and is ideal for intraprotein studies. The R_0 values reported by Wu and Brand for 14 combinations with Trp as the donor and a dye as the acceptor range from 12 to 40 Å, which also represents a good estimate of the viable FRET range with dye acceptors.[30] From this range one can generalize that Trp residues almost anywhere within a "smaller" protein (diameter < 3 nm; $M_r < 30\,000$) will probably function as either a donor or an acceptor for an appropriate dye implanted within the protein structure. Indeed, if several Trp residues are present they will contribute to FRET to different extents on the basis of their separation distance. In view of the relative ease of introducing mutations into proteins, it should not be difficult to design modules with fluorescent residues for appending onto proteins of interest. These modules could function as efficient donors with large absorption cross sections to augment a FRET-based biosensing protein or as a tandem donor for a fluorescent protein or in a light-harvesting complex. With the growing interest in the structure and function of proteins, these endogenous fluorophores clearly remain underutilized.

3.2. Fluorescent Proteins

Fluorescent proteins (FPs) are being used increasingly in FRET systems, and the technologies and materials are continually improving. There are clear conceptual benefits to a fluorophore that is genetically appended onto the gene coding for a protein of interest to create a fluorescent chimera: the fluorophore and protein can then be co-expressed intracellularly and, when visualized, reveal the location and relative expression level.[14,20,21] The green fluorescent protein (GFP; Figure 9) derived from the jellyfish *Aequorea victoria* is the prototypical fluorophore of this protein family and has been used to revolutionize many aspects of cell biology.[120] Tsien provides an excellent monograph on this protein and its photophysical function.[20]

Figure 9. Ribbon structure of the green fluorescent protein (GFP).

The GFP was first described more than 40 years ago but was not cloned until the early 1990s. Key to its widespread use was the demonstration that this gene could be expressed in other organisms, since the coding sequence alone contains everything needed for the chromophore to mature and function.[20] Key internal residues are modified during maturation to form the *p*-hydroxybenzylideneimidazolinone chromophore, located in the central helix and surrounded by 11 β strands (β-can structure).

Many GFP variants exist that differ in protein and chromophore structure and hence also in their absorbance and emission profiles.[20] Through mutation and selection, a more enhanced and stable GFP was produced, as well as blue, cyan, and yellow fluorescent proteins (BFP, CFP, YFP).[21] The red fluorescent protein (DS Red) was cloned in 1999 and revealed to be an obligate tetramer that matured slowly from green to red.[21,121,122] Tsien and co-workers developed a monomeric red fluorescent protein (mRFP) and various other red fluorophores, which they named after the fruit colors they resemble.[21,123] Other colors of FPs have been cloned from coral; these also appear to be tetrameric.[21] Figure 10 shows the absorption and emission profiles of representative FPs.

There are also commercially available FPs, such as the red/green series of phycobilisome-derived PBXL fluorophores.[124] These are stabilized multichromophore supramolecular protein complexes that can be linked to proteins. The increased number of fluorophores provides significantly higher sensitivity.

FPs are primarily being used for in vivo labeling of cells. FPs encoded in plasmids are available that are optimized for cloning proteins at either the N- or C-terminal. The plasmids allow controlled expression in a variety of cells and organisms including bacteria, yeast, and eukaryotes. The quantum yields of these proteins are generally good, ranging from 0.17 for a BFP to around 0.79 for a wild-type GFP, and largely depend upon which mutations are present and the final chromophore structure.[21] These benefits do, however, come with liabilities. Most FPs are large ($M_r \approx 25$ to 30 kD and larger); appending a protein of this size onto another protein while maintaining the desired function can be problematic.[24] An FP can also be placed in the center of a protein or on the intra- or extracellular membrane; however, the correct folding, insertion, and fluorescence are never guaranteed. FPs that form dimers and tetramers can compound issues of creating bifunctional chimeras.[21,121] It can take several hours for FPs to mature and for the final chromophore to be formed, and the absorption and emission may shift during this process. These proteins are also susceptible to pH, temperature, O_2 concentration, and other environmental conditions.[20] Although many FPs may be sensitive to photobleaching, this need not be a liability, since advanced imaging techniques such as fluorescence recovery after photobleaching (FRAP) can exploit these phenomena.[125] As can be seen in Figure 10, the generally broad absorption/emission profiles of an FP may preclude multiplex analysis.

The strategy of Tsien and co-workers for FRET-based FP indicators created a new class of genetically encoded sensors for monitoring intracellular analytes.[126,127] The original constructs (termed "cameleons") were designed to sense calcium

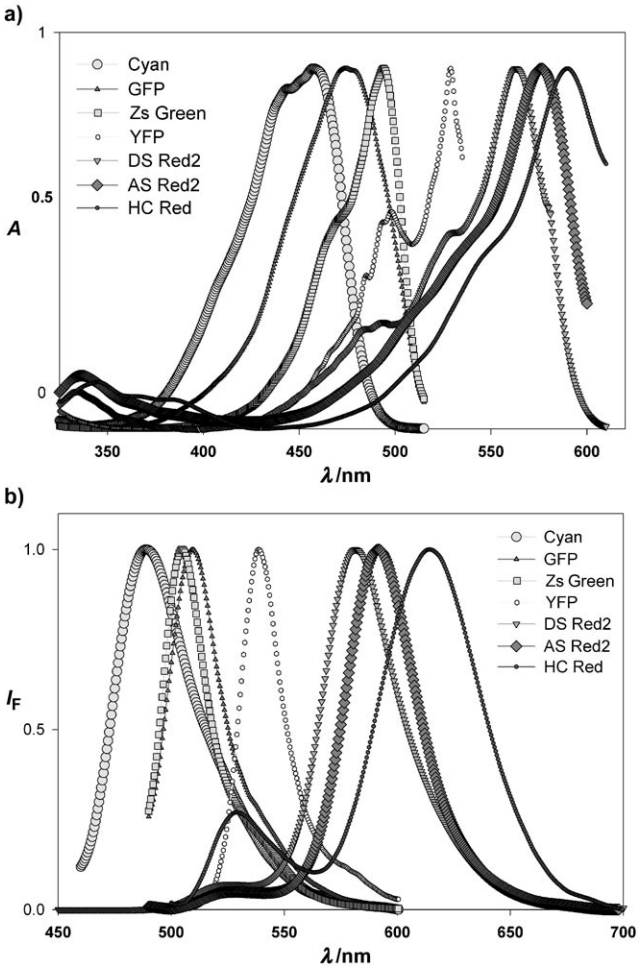


Figure 10. a) Normalized absorption and b) fluorescence profiles of representative fluorescent proteins: cyan fluorescent protein (cyan), GFP, Zs Green, yellow fluorescent protein (YFP), and three variants of red fluorescent protein (DS Red2, AS Red2, HC Red). Figure courtesy of Clontech.[14]

and consisted of linear fusions of BFP or CFP donors and enhanced GFP or YFP acceptors, which flanked calmodulin and the calmodulin-binding peptide.[127] Upon Ca^{2+} binding, calmodulin wraps around the peptide, so that the distance between the flanking FPs is reduced and the FRET increased. Following this strategy, the group of Frommer developed an elegant series of intracellular sensors that consist of FPs fused to the N- and C-termini of bPBPs.[128–130] The prototype consisted of MBP with an enhanced CFP (ECFP) donor fused to the N-terminus and a YFP acceptor fused to the C-terminus (Figure 11).[129] MBP belongs to the superfamily of hinge-binding proteins. Upon binding maltose, it undergoes a conformational change around the central hinge. This movement causes the two FPs to move closer together, thus altering the FRET efficiency and allowing transduction by a change in emission ratio of the donor and acceptor (Figures 11 and 12).

Additional sensors target glucose and ribose; however, the FPs in these sensors move apart in response to binding, thus resulting in a decrease in the D/A fluorescent ratio.[128,130] The overlap of the absorption and emission spectra of

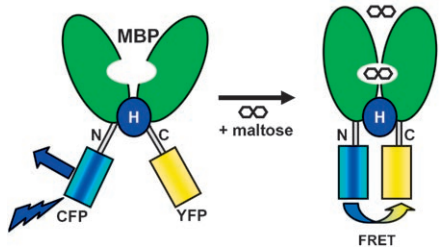


Figure 11. Modified MBP fluorescent indicator. ECFP as donor was fused to the N terminus of MBP, and YFP as a FRET acceptor was fused to the C terminus. H indicates the portion of protein functioning as a hinge between the two lobes of the MBP. The central binding pocket of the MBP is located between the two lobes. In the abscence of maltose, the two FPs are at their maximum distance from each other and FRET is minimal. Upon binding maltose, the MBP undergoes a conformation change that brings the two FPs into close proximity and increases FRET, which can be monitored by the change in ratio of the YFP and CFP emission (see Figure 12).[129]

multiple-FP fusions results in small dynamic changes in FRET configurations; therefore sensitive optical equipment and spectral deconvolution are necessary. Biosensors that are based on this tandem-FP consensus design have now been developed to target kinases, lipases, various intracellular second messengers, and proteases.[120]

Rice created a kinesin C-terminal GFP fusion and labeled the kinesin with tetramethylrhodamine to allow FRET monitoring of the conformational changes of the protein upon binding nucleotides.[131] Hoffman et al. demonstrated a novel combination of CFP and the FlAsH system to label a G-

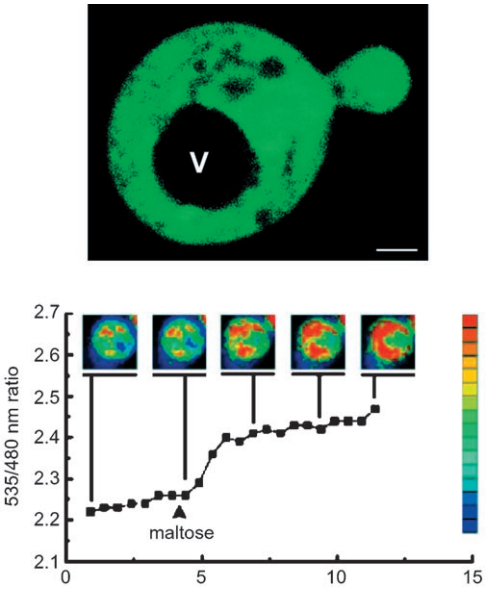


Figure 12. a) Confocal image of a maltose-FP sensor expressed in yeast. Fluorescence is detected in the cytosol but not in the vacuole. Scale bar = 1 μm. b) Changes of the maltose concentration in the cytosol of yeast that expresses a maltose sensor with a K_d value of 25 μM. The graph indicates emission ratio as a function of maltose uptake for a single yeast cell. Figure generously provided by W. Frommer, Stanford University; reproduced with permission of the National Academy of Sciences USA.[129]

protein-coupled receptor system.[24] Dual labeling of the same receptor with CFP and YFP maintained receptor activation but disrupted downstream signaling. Replacing YFP with a FlAsH dye allowed normal downstream signaling.[24]

Those interested in using these proteins can now consult several guides.[21,132] The increased interest in utilizing FPs for FRET and other intracellular applications has stimulated their continual improvement, and enhanced GFPs that are more tolerant to pH and environment have been created.[20] Through mutational selection, monomeric RFPs have been developed from the original tetramers and dimers.[123,133] For optimal FRET pairing, Nguyen and Daugherty developed a CFP–YFP pair that exhibited a 20-fold change in the FRET signal ratio (compared with a 3-fold change for the the original construct).[134] This new D/A pair should enable FP FRET sensors in which the donor and acceptor have less than optimal configuration. FPs from different species have also been cloned with new colors and interesting properties such as photoconversion.[135]

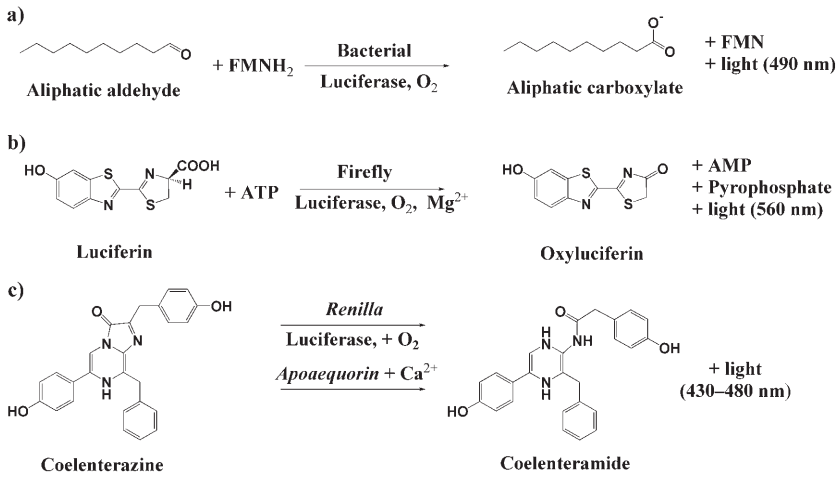
3.3. Enzyme-Generated Bioluminescence

Enzyme-generated bioluminescence (BL) is a natural phenomenon found in certain beetles, bacteria, and marine species. In BL, the substrate luciferin is oxidized by a luciferase enzyme in the presence of O_2 and sometimes a cofactor such as ATP.[136–139] The oxidation of luciferin yields an excited-state molecule that decays with light emission (Scheme 4). BL has found applications as a reporter in many bioassays.[140–143] The light emitted from a BL system can also be exploited for energy transfer to an appropriate acceptor.[144–147] This process, known as bioluminescence resonance energy transfer (BRET), is a variant of FRET and is similarly efficient for D/A separation distances from 10 to 100 Å.[141] Luciferase acts as the donor in a BRET system, and the acceptor is usually GFP, which is also the physiological acceptor in luminescent organisms.[148,149] The principal advantage offered by BRET is that no excitation light source is required to excite the donor, which avoids problems such as light scattering, high background noise, and direct acceptor excitation.[146] Additionally, since the donor or both the donor and acceptor can be co-expressed in the cell as fusion proteins and the excitation follows a localized event (luciferin delivery), the target of interest can be excited specifically, which is especially important for applications in vivo.[145]

BRET reporter pairs have been utilized for in vivo monitoring of protein–protein interactions including the interactions between circadian clock proteins,[144] insulin receptor activity,[150–152] and the real-time monitoring of intracellular ubiquitination.[145,153] Whereas the acceptor is usually conserved as GFP or one of its variants, a variety of related donor enzymes have been employed. The most commonly exploited luciferases are the terrestrial and marine bacterial luciferases and the eukaryotic firefly and *Renilla* (Sea Pansy) luciferases (Table 2). Luciferases catalyze the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde in the presence of O_2 to yield blue light (Scheme 4a).[154,155] Because $FMNH_2$ is rapidly oxidized in air, this luciferase cannot provide continuous emission, but instead generates only short bursts of light.[137] Also, since these genes are not easily expressed in mammalian cells, bacterial luciferases have found limited applications. No BRET application of bacterial luciferase has been reported to date.

The firefly luciferase/luciferin pair is the most commonly exploited BL reporter system. This luciferase catalyzes the oxidation of luciferin in the presence of ATP with emission of green-yellow light (Scheme 4b).[156] The light emitted initially is highly intense, but then decays to a sustained low-intensity luminescence. The addition of coenzyme A can help to yield a more-stable, high-intensity luminescence that decays over several minutes.[157] Caged luciferin, which is designed for intracellular delivery, is commercially available.[158] Once inside the cell, this luciferin can be activated either by UV light or by the action of intracellular esterases. The firefly luciferase/luciferin system is probably the best candidate for a BRET-based donor, as it shows a high quantum yield (0.88) and is easily expressed in *E. coli*. However, as its emission maximum is around 560 nm, GFP and some of its variants are not suitable as acceptors. Alternative acceptors such as Cy3/Cy5 and the fluorescent protein DS Red have already been used with this protein donor to monitor antigen–antibody binding[159] and protein–protein interactions.[160]

Renilla luciferase (RLuc) catalyzes the oxidation of coelenterazine to coelenteramide with the emission of blue light (Scheme 4c).[138] Although the quantum yields of RLuc are low (0.07) in comparison with those of firefly luciferase, the assays are simpler to perform as cofactors are not required. Unfortunately, RLuc exhibits a certain amount of autoluminescence, which results in a less sensitive assay. Even with this limitation, the *Renilla* luciferase/coelenterazine system is the first and probably most exploited donor for BRET systems.[144] Exam-



Scheme 4. Bioluminescent substrates and enzymatic reactions of several common luciferases: a) the aliphatic aldehyde substrate of bacterial luciferase; b) structure and reaction of luciferin, the substrate of firefly luciferase; c) colenterazine, the substrate for *Renilla* luciferase and also part of *apoaequorin*.

Table 2: Characteristics of common enzymes that catalyze bioluminescent and chemiluminescent reactions, along with their substrates.

Enzyme	Gene	M_W [kDA]	Substrate	Cofactor(s)	λ_{em}	Notes	References
			Bioluminescence				
bacterial luciferase (*Vibrio*, *Photobacterium*, *Xenorhabdus* genera)	*Lux*	80 (dimer)	aliphatic aldehyde	$FMNH_2$, O_2	490	limited applications	[136, 154, 155]
firefly luciferase (*Photinus pyralis*)	*Luc*	61 (monomer)	luciferin[a], caged luciferin[b]	ATP	560	coenzyme A increases luminescence; BRET acceptors: Cy3/Cy5, DS Red.	[156–160]
Renilla luciferase	*Ruc*, *hRluc*	35 (monomer)	coelenterazine, coelenterazine (*h*, *n*),[c] coelenterazine *cp*, *f*,[d] DeepBlueC	none	475 442–473 395	autoluminescence	[138, 147, 164, 165]
Gaussia luciferase	*hGluc*	20 (monomer)	coelenterazine	none	480	[e]	[167–169]
Aequorin (from jellyfish *Aequorea victoria* or recombinant)		22 (monomer)	coelenterazine, *f*, *h*, *hcp*, *cp*, and *n*	Ca^{2+}	445–475	[f]	[139, 172]
			Chemiluminescence				
horseradish peroxidase (*Armoracia rusticana*)		44 (glycoprotein)	luminol, isoluminol, lumigen, acridan	H_2O_2[g]	411–425	[h]	[183–185]
alkaline phosphatase (*Pandalus borealis*)		106 (homodimer)	1,2-dioxetanes (lumigen, Lumi-Phos, CDP-Star)		480, 530	[i]	[191, 192]

[a] D-(−)-2-(6′-hydroxy-2′-benzothiazolyl)thiazoline-4-carboxylic acid. [b] Available from Molecular Probes, Promega. [c] Molecular Probes, Biotium. [d] Available from Perkin Elmer (BRET^2). [e] Available from Prolume and NEB. [f] Available from Lux Biotech. and Molecular Probes. [g] Enhancers: luciferin, fluorescein, phenolic compounds. [h] Aureon Biosystems, Vector Labs and Alpha Innotech. [i] Michigan Diagnostics.

ples of its use include the monitoring of the dimerization of the β-adrenergic receptor[161,162] and the binding of the insulin-like growth factor II to the insulin-like growth factor binding protein in living cells.[163] Two new *Renilla* genes (*hRluc*) that are optimized for expression in mammalian cells have also been created (Table 2). The emission of *Renilla* luciferase can also be modulated by choosing the appropriate coelenterazine substrate, and several analogues with different emissions are available.[164,165]

Perkin–Elmer has developed a proprietary BRET2 assay that utilizes *Renilla* luciferase, a coelenterazine substrate named DeepBlueC (emission at 395 nm), and an optimized GFP2 acceptor. This configuration functions like a standard BRET assay, but has greater spectral resolution between the donor and acceptor pair.

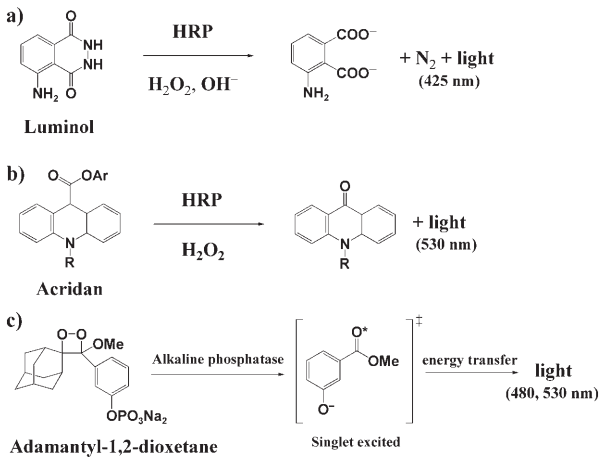
Another luciferase from *Gaussia* (hGluc), has been optimized for expression in both bacterial and mammalian cells.[166,167] With a molecular mass of only 20 kDa (compared with 35 kDa for *Renilla*), hGluc displays spectral characteristics similar to Rluc while also addressing problems associated with steric constraints in chimeric fusions. *Gaussia* luciferase expressed in mammalian cells generates up to 1000-fold brighter light than native *Renilla*.[168] Although hGluc has been employed as a reporter label for following DNA hybridization[167] and for monitoring bacterial cells,[169] there are no reports of its use as a BRET donor. Other isolated luciferases include the 19-kDa luciferase from the luminous shrimp *Oplophorus gracilirostris*, which catalyzes the oxidation of coelenterazine and emits light at 454 nm with high quantum yield at temperatures up to 40 °C.[170]

Aequorin, derived from jellyfish, is a Ca^{2+}-sensitive bioluminescent photoprotein consisting of the luciferase apoaequorin complexed to its coelenterazine substrate (Scheme 4c)[171]. The blue bioluminescence of *Aequorin* is triggered by Ca^{2+} ions; hence, its principal application is as a reporter for Ca^{2+} ions.[139,172] *Aequorin* has been employed as a BRET donor to monitor the interaction between Streptavidin (fused with *Aequorin*) and a biotin carboxyl carrier protein (fused with an EGFP acceptor).[173] In a modified BRET assay, the bioluminescence of biotinylated *Aequorin* was quenched by Dabcyl or QSY-7-labeled avidin upon exposure to Ca^{2+} ions.[174]

In general, BRET systems with the described enzymatic donors have been exploited mostly for in vivo assays. However, it is foreseeable that applications such as biosensors that do not require an excitation source can be developed.

3.4. Enzyme-Generated Chemiluminescence

Enzyme-generated chemiluminescence (CL) is closely related to BL, with the difference being that the luminophore in this case is a synthetic substrate that is excited through an enzymatically catalyzed reaction.[143] Scheme 5 and Table 2



Scheme 5. Chemiluminescent substrates and the enzymatic reactions of horseradish peroxidase (HRP) and alkaline phosphatase. a) Luminol; b) Acridan (also available as an ester); c) Adamantyl-1,2-dioxetane (substrate for alkaline phosphatase and other enzymes).

describe some common CL substrates along with their processing enzymes. In general, the quantum yields for CL are lower than for BL. CL has found broad applications as a sensitive reporter system in drug screening, capillary electrophoresis, and immunoassays.[140–143,175–177] Although examples of chemiluminescent resonance energy transfer (CRET) are known,[178–182] this concept remains relatively underexplored. Akin to BRET systems, CL labels are potential donors in CRET-based assays.

Horseradish peroxidase (HRP) is probably the most commonly used enzyme for CL detection. Although a variety of chemiluminescent substrates exist for this enzyme, luminol and its luminogenic derivatives remain the most popular (Scheme 5a).[183–185] In the presence of hydrogen peroxide, HRP oxidizes luminol to give a luminescent species that emits blue light (425 nm). Luminol is usually employed in conjunction with an enhancer, such as luciferin, fluorescein, or a phenolic compound.[186,187] Exposing acridan substrates to HRP generates luminescent acridinium ester intermediates, which decay with emission of yellow light (530 nm) at a higher luminescent intensity than that of luminol (Scheme 5b).[188–190] Alkaline phosphatase is also commonly used to catalyze the oxidation of 1,2-dioxetanes as a luminogenic substrate (Scheme 5c).[191,192] Since 1,2-dioxetanes are inherently unstable four-membered cyclic peroxides, more-stable substrates such as adamantyl-1,2-dioxetane phosphate were developed. Dioxetanes are usually delivered in combination with proprietary enhancers that increase the stability and luminosity, and also expand the spectral range through energy transfer to another fluorophore; in this way, two emission wavelengths are obtained (480 and 530 nm). Dioxetanes can also be luminogenic substrates for other enzymes such as β-D-galactosidase, β-glucosidase, β-glucuronidase, arylesterase, arylsulfatase, and neuramidase.

Although CRET systems have been utilized predominantly as reporters, the large number of recombinant enzymes available, coupled with the low cost of commercial substrates and the ability to control the emission wavelength, opens up the possibility of exploring CRET applications in biosensors.

4. Inorganic Materials

4.1. Metal Chelate Complexes and Long-Lifetime Dyes

Luminescent lanthanides are the most prominent class of long-lifetime dyes used for energy-transfer applications in biophysical research. Selvin's group has been at the forefront of developing these probes for biological studies.[193] Four lanthanides emit in the visible region: terbium, europium, samarium, and dysprosium. Because of the high intensity of their emission, Tb and Eu cations are most commonly used.

For biophysical applications, lanthanide cations are typically complexed within a chelate ligand, whose design must fulfill several functional requirements (Figure 13): 1) The lanthanide ion must form a tightly bound complex with the ligand, so that high thermodynamic and photochemical stability is achieved and the lanthanide ion is shielded from the quenching effects of the surrounding solution. Chelate ligands often take the form of polyaminocarboxylates, pyridines, or salicylic acid derivatives.[193,194] 2) Relative to common dyes, lanthanide ions have very low extinction coefficients ($\approx 1\,M^{-1}cm^{-1}$), which makes them difficult to excite directly. Thus, the chelate label must contain an organic chromophore in close proximity to the ion which functions as a light-harvesting antenna or sensitizer. The sensitizer molecule absorbs incident light and transfers this energy to the lanthanide ion. 3) The chelate label should possess a reactive group to allow bioconjugation.

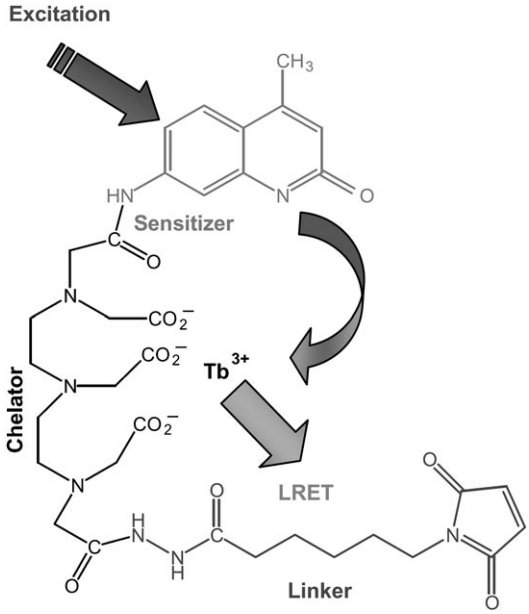


Figure 13. Structure of the LanthaScreen Tb probe from Invitrogen with the functionalities highlighted. The linker group is typically either an NHS ester or isothiocyanate/maleimide group.

Research continues on the improvement of antennas and development of methods for the direct coupling of antennas and chelators to the termini or side chains of nascent peptides.[195,196] The currently available linkers for coupling these probes are relatively long and flexible, which leads to some uncertainty in the analysis. Direct attachement of the probes should improve the accuracy of measurements of the D/A distance. Sources for lanthanide probes include CIS-Bio International (cryptate-based probes), Perkin–Elmer, Invitrogen (LanthaScreen), and Amersham Biosciences (europium–TMT chelates).

Long-lifetime donors (fluorescent lifetime $\tau > 100$ ns to several ms) have a number of technical advantages over conventional fluorescent dyes ($\tau = 1$–5 ns). The principal benefit arises from the ability, through time-resolved measurements, to eliminate background fluorescence (from direct excitation of dyes, scattering, and autofluorescence from cells and biomolecules), thereby dramatically improving sensitivity. Lanthanide probes also possess multiple distinct, sharp emission bands and large Stokes shifts, so that D/A emission can be detected far from the excitation wavelength (Figure 14). Together these properties allow lanthanide probes to be coupled to a wide range of acceptor dyes. Terbium, for example, has good spectral overlap with fluorescein, rhodamine, and Cy3. Selvin's review article has a list of matching dyes along with their corresponding R_0 values.[193]

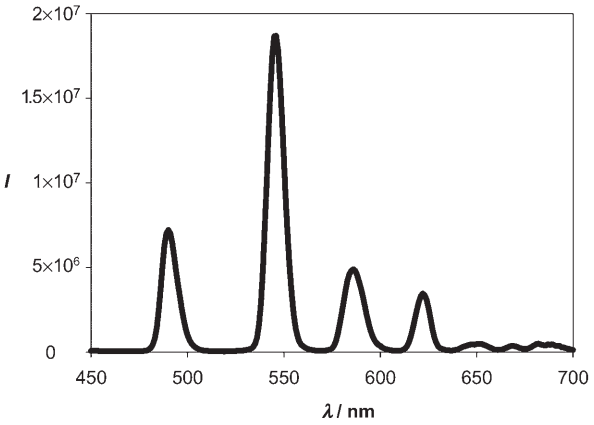
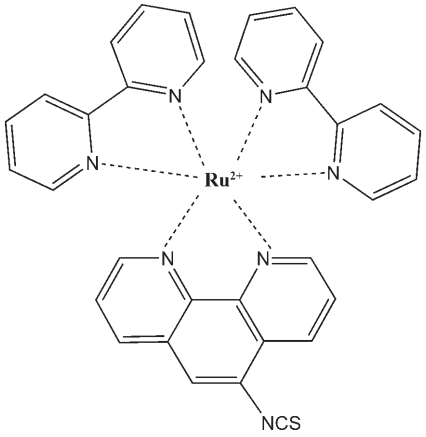


Figure 14. The unique sharp emission profile of the LanthaScreen Tb probe ($\lambda_{exc,max} \approx 343$ nm).

Resonance energy transfer using lanthanide donors is (more correctly) referred to as luminescent resonance energy transfer (LRET), since technically lanthanide emission is not considered fluorescence. However, it originates from the same electric dipole transitions as conventional organic dyes and is therefore governed by the same r^6 distance dependence as for FRET. The high quantum yields of the lanthanide probes (0.1–0.4) translates into R_0 values up to 100 Å. Care should be taken in the determination of the spectral overlap, since some emission bands arise from both magnetic and electric dipole transitions, whereas only the electrical transitions allow significant energy transfer.[197] Time-based measurements require more-complex equipment than that needed for steady-state measurements. However, because the dyes have long lifetimes (μs to ms), the instrumentation is typically less costly than that required for measurements with conventional dyes (ns lifetimes). In fact, many microtiter well-plate readers are available that allow gated lifetime measurements in this timescale.

LRET studies with lanthanide probes typically use conventional dyes as acceptors.[193] Lanthanide-based LRET has been used to study the activity of enzymes such as telomerase, caspase, helicase, and phosphatase.[198,199] An Eu–Cy5 D/A pair has also been used in high-throughput screening of potential antimicrobial drugs.[200] The same Eu-Cy5 pair was also used for competitive immunoassays of urinary albumin and noncompetitive assays of morphine.[201,202] Tsourkas et al. developed molecular beacons with Tb- and Eu-labeled DNA donors and demonstrated that time-resolved measurements with this LRET pair required neither a quencher nor a hairpin structure on the lanthanide-labeled probe.[203] An LRET system with a Tb donor and a Cy3 acceptor has also been used to monitor DNA hybridization.[204] Lanthanide probes have also been used in elucidating biological structures, for example, for measuring conformational changes in ion channels and enzymes, monitoring transmembrane signal transmission through voltage-sensitive segments within a functional potassium channel, and measuring distances across thin muscle filaments.[193,205–207]

Sigma–Aldrich offers a series of reactive ruthenium complexes (Scheme 6) that were originally developed by



Scheme 6. Structure of the commercially available ruthenium complex that is typically used in long-lifetime fluorescent studies.

Lakowicz and co-workers as anisotropy labels for measuring the rotational dynamics of proteins.[208,209] These Ru complexes have lifetimes of approximately 500 ns and are thus closer to organic dyes than lanthanides. As with the lanthanide probes the main advantage is the ability to monitor fluorescence selectively after the background fluorescence has decayed. Ru complexes have relatively small extinction coefficients ($14\,500\ \text{M}^{-1}\text{cm}^{-1}$) and low quantum yields (0.05), but these disadvantages are again offset by their long lifetimes, high photostability, fairly large Stokes shift, and

absorption across almost the entire visible spectrum (Figure 15). Ru complexes have been applied as LRET donors in direct and competitive immunoassays for human serum albumin.[209] In another case, an environmentally

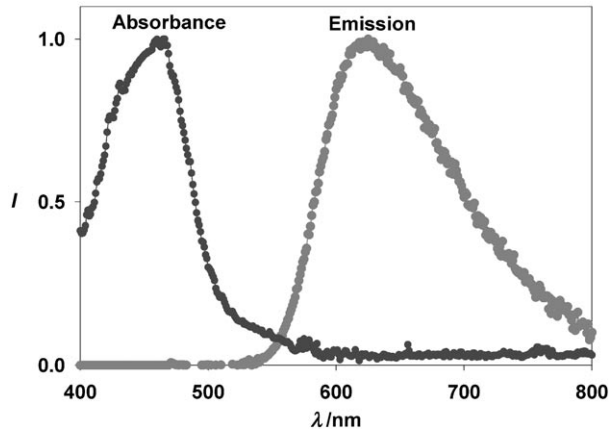


Figure 15. Absorption and emission profiles of the ruthenium complex shown in Scheme 6.

sensitive Sudan III diazo acceptor dye was coupled to a Ru complex in silica gel and used for the LRET-based detection of CO_2.[210] In a rare example of the use of the complex as an acceptor, a glucose-binding protein was labeled with an environmentally sensitive acrylodan dye and a Ru complex.[211] The acrylodan was affected indirectly by glucose (which altered the protein conformation), whereas the Ru complex was not affected and thus served as an internal standard for ratiometric measurements.

Other types of materials for LRET applications have hardly been explored. All long-lifetime probes could quite easily be paired with dark quenchers. However, time-gated detection obviates this. As time-resolved fluorimeters become more accessible, long-lifetime probes will see increased use in LRET assays, driven mainly by the dramatic increase in signal-to-noise ratio afforded.

4.2. Gold, Metal, and Silicon Nanoparticles

4.2.1. Gold

Gold nanoparticles (NPs) are increasingly used in FRET-based applications, mostly because of their exceptional quenching ability. Gold and other noble metals have unique properties, such as plasmon resonances in the visible range (typically with large extinction coefficients around $10^5\ cm^{-1}M^{-1}$), stable, unfluctuating signal intensities, and resistance to photobleaching. Daniel and Astruc provide an excellent review of almost all properties of gold NPs,[212] including the somewhat murky distinction between clusters and colloids (the later have size polydispersity).

Besides standard FRET considerations, the size and shape of the gold NPs also play an important role in FRET systems. Detailed studies have characterized the fluorescence quenching of dyes attached at a fixed distance from the surface of various sized gold NPs (1–30 nm) as well as dyes attached at varying distances (2–16 nm) from the surface of 6-nm gold NPs.[213] Almost all the gold NPs were found not only to increase the nonradiative rate of decay of the dye, but also to decrease the radiative rate—even 1-nm gold NPs were capable of greater than 99% quenching efficiency.

Gold NPs can be produced in various sizes by using either the citrate-reduction (diameter 16–147 nm) or the Brust–Schiffrin method (diameter 1.5–5.2 nm).[212] One of the intrinsic benefits of using gold NPs is that biomolecules containing exposed thiol groups can be attached to the NPs directly through gold–sulfur bonds. Gold NPs can also be treated with sulfur-containing ligands that possess distinct terminal groups (e.g., carboxylic acids or amines) that in turn can be used for subsequent bioconjugation. Alternatively, Nanoprobes offer 1.4-nm gold nanoclusters that are activated with either a single succinimidyl ester or maleimide.

Gold NPs have been used successfully in FRET applications with molecular beacons for the sensing of DNA (Figure 16). These were 100-fold more sensitive than previous

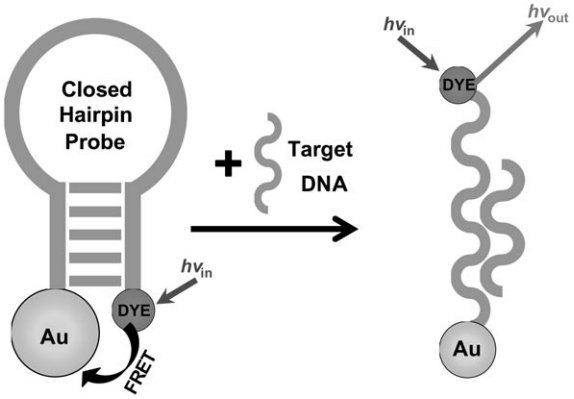


Figure 16. Schematic of a gold nanoparticle probe: In the closed hairpin structure, the D/A pair are in close proximity and the fluorescence in quenched.[214] Hybridization of the target single strand DNA opens up the structure of the molecular beacon, which increases the distance between the gold NP and the dye and results in a significant increase in fluorescence.

dye combinations.[214,215] The research group of Krauss developed a system in which molecular beacons are immobilized onto gold surfaces.[49,216] Both surface- and NP-based molecular beacons using organic dye donors demonstrate a high sensitivity for single base-pair mismatches. Seidel et al. demonstrated a FRET-based immunoassay for the detection of the pesticide atrazine by using gold-coated well plates (Figure 17).[217] Recently, gold NPs have also been tested as quenchers for semiconductor QDs (see Section 4.3). The hybridization of two complementary pieces of single-strand DNA, one attached to a QD and the other coupled to a 1.4-nm gold NP, was monitored by FRET (Figure 18).[218,219] The formation of nanoscale assemblies between oppositely charged QDs and gold NPs in solution has also been monitored by FRET.[220] An inhibition assay with streptavidin-coated QDs and biotin-functionalized gold NPs has also been reported (Figure 19).[221] The results from these studies

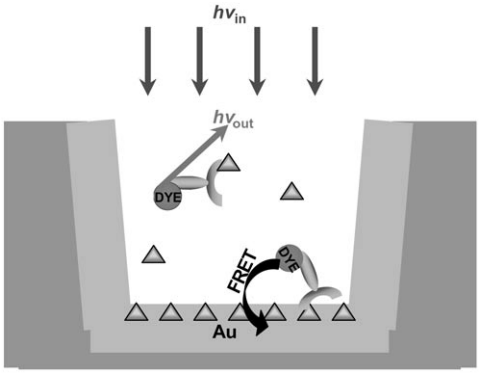


Figure 17. Gold-coated well plates for the competitive immunoassay detection of atrazine.[217] The binding of dye-labeled antibodies to the atrazine immobilized on the gold surface results in FRET quenching of the dye. Free atrazine in solution competes with the toxin on the gold, prevents the binding of the antibody to the surface, and thus increases the fluorescence.

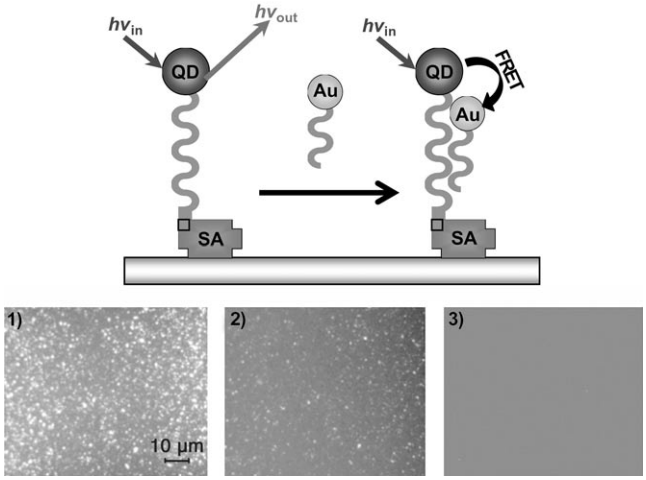


Figure 18. Detection of DNA hybridization by quenching upon binding of a gold-labeled single strand of target DNA. 1–3) Fluorescence signal of the surface after introduction of the gold target; $t = 0$ min (1), 5 min (2), 15 min (3). Images generously supplied by T. Melvin and reproduced with permission from the Royal Society of Chemistry. [218]

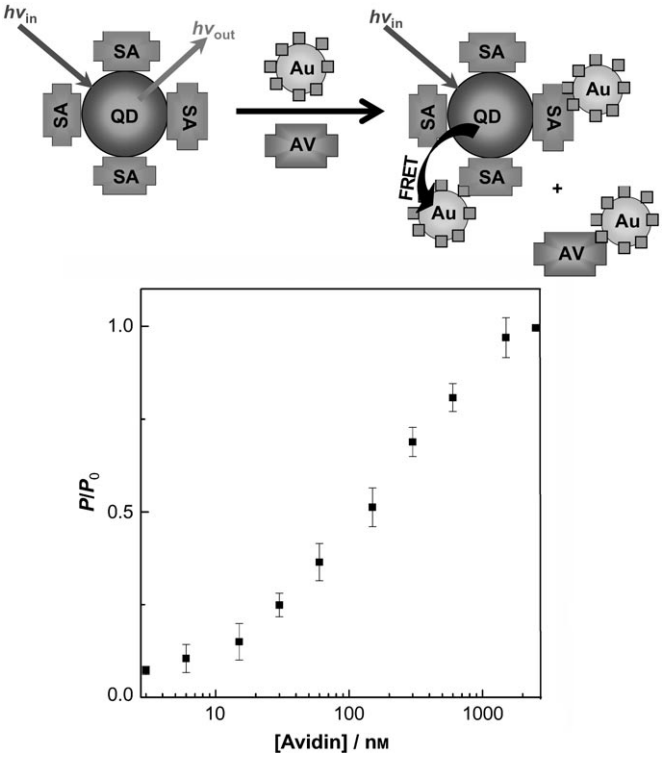


Figure 19. Top: Competitive inhibition assay for the detection of avidin on the basis of quenching of QDs by gold nanoparticles. Binding of the biotin-functionalized gold particle brings it into proximity of the strepavidin-labeled QD, which results in FRET and loss of QD photoluminescence. Avidin in solution competes with the strepavidin-labeled QDs for the biotin-gold particles and thus changes the FRET. Bottom: The resulting dose response for the assay. Figure generously supplied by E. Ohand; reproduced with permission from the American Chemical Society.[221]

suggest that such FRET configurations have tremendous potential. The main advantages are the lower background signal, the improved sensitivity, and the ability to label both the gold NP and QD with multiple biologically active groups.

Gold is typically used for its quenching abilities; another possible use in which highly fluorescent gold QDs are used was described recently by Dickson and co-workers.[222,223] Much like their semiconductor counterparts, these gold QDs have size-tunable emission maxima, which shift to longer wavelengths with increasing nanocluster size. Fluorescent gold QDs can be used in FRET applications as both donors and acceptors; also, since the surface is stabilized with poly(amidoamine) dendrimers (PAMAM), the free amines on the dendrimer could be used in bioconjugation. It is quite clear that the use of gold NPs and surfaces for FRET measurements is still in its infancy, but many new applications can be expected in the near future.

4.2.2. *Metal and Silicon Nanoparticles*

Interest in single-molecule optoelectronic materials has driven research into the fluorescence properties of small metallic NPs.[222,224] Clusters constituted of just a few noble-metal atoms show interesting emission properties, provided they are appropriately stabilized.[225,226] The fluorescence of noble-metals NPs can be intense; however, it is difficult to control the emission wavelength.[227] Besides gold NPs, silver NPs have also been shown to have interesting optical properties such as shape-dependent absorption and highly intense fluorescence.[228–233] Copper nanoparticles have been less studied; they display a large plasmon resonance peak in the visible range and interesting nonlinear optical properties.[234–236]

Silicon NPs have equally interesting optical characteristics, such as bright size-dependent photoluminescence and broad excitation spectra.[237–240] Because of their brightness and resistance to photobleaching, Si NPs have been investigated as fluorescent tags for DNA[241,242] and potentially nontoxic alternatives to semiconductor materials for in vivo

imaging.[243] The synthesis and stabilization of Si NPs remains tricky, although viable methods exist.[244,245] Wiesner and co-workers developed hybrid nanoparticles with a fluorescent core and a silicon shell which they refer to as CU dots (Cornell University).[246] These hybrids are synthesized by covalent conjugation of dye molecules to a silicon precursor and condensation to form a dye-rich core. Finally, silicon sol–gel monomers are added to form a denser outer silicon network. Because of their photostability, tunability, and ease of surface modification, applications of Si-based NPs as FRET donors can be expected.

Extensive studies on metal NPs coated with fluorescent dyes have confirmed plasmonic enhancement effects.[247–251] This effect involves energy transfer from the excited-state fluorophore to the plasmon resonance of the proximal metal surface/particle, which results in significantly different fluorophore excitation and emission properties.[249,251] Plasmon enhancement also decreases the excited-state lifetime of the fluorophore, which may increase stability by reducing photobleaching. The type of metal, size of the NP, and the fluorophore all have an influence in this complex process, but the general effect is that the quantum yield of the fluorophore increases dramatically, particularly for fluorophores with low quantum yield.[253–259] For plasmon enhancement to function, the spacing between fluorophore and metal must be carefully tuned.[256–259] The effect has already been exploited to increase the FRET efficiency between DNA-bound fluorophores,[260] and it is just a matter of time before more viable configurations are found.

4.3. Semiconductor Nanocrystals

Pioneering studies demonstrated that colloidal luminescent semiconductor nanocrystals or QDs could be used for the detection of proteins or DNA.[261,262] Extensive reviews can be found in references [263–268]. QDs have several unique intrinsic photophysical properties which make them attractive biolabels: relatively high quantum yields, molar extinction coefficients 10 to 100 times those of organic dyes, as well as high resistance to photobleaching and chemical degradation.[263–265] In direct comparison with organic dyes, several properties of QDs stand out: 1) size-tunable photoluminescent emission; 2) broad absorption spectra and large Stokes shifts, which allow excitation of mixed QD populations at a wavelength far from their emission wavelengths (Figure 20).[263–265,269,270] For FRET applications in particular, this means that QDs can be size-tuned or "dialed in" to give better spectral overlap with a particular acceptor dye (Figure 21).[271] As the spectral overlap increases, there is a proportional increase in the value of R_0, which, together with the high quantum yield of the QDs, permit FRET systems with longer separation distances. Since QDs can be excited at almost any wavelength below their emission wavelength, an excitation wavelength can be chosen that corresponds to the absorption minimum of the acceptor so that direct excitation is minimized.

QDs for biological assays are commercially available (Quantum Dot Corporation and Evident Technologies).

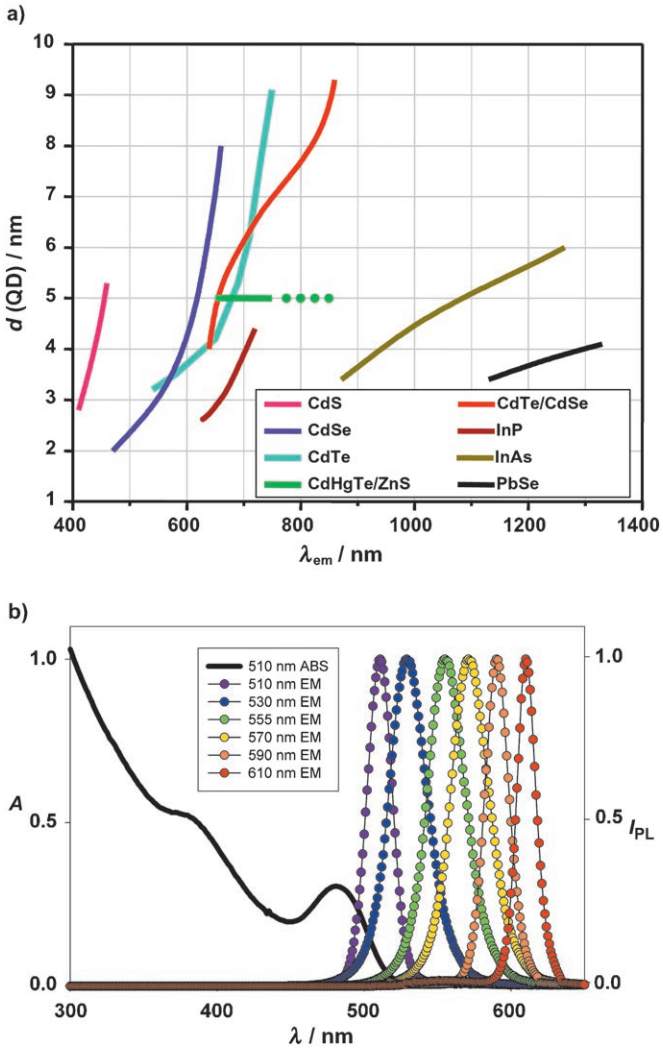


Figure 20. a) Correlation of emission maxima with the size of QDs composed of different binary and ternary semiconductors. b) Absorption and emission of six different QDs (in buffer) that have been utilized in several assays.[263] The black line shows the representative absorption of the QDs that emit at 510 nm. Note that the absorption increases steadily towards the UV. Figure generously provided by X. Michalet, UCLA, and reproduced with permission from reference [265].

These materials are available precoated with avidin or other proteins to facilitate bioconjugation. There are also several detailed monographs describing QD synthesis.[227,270,272–274] The best available QDs for biological applications consist of a CdSe core material coated with a ZnS shell (Figure 22). The shell passivates the core, protects it from oxidation and leeching, and at the same time significantly improves the photoluminescence.[263–265,270] Since QDs are typically synthesized from insoluble salts, they are also not water-soluble. Therefore, the native organic ligands used for synthesis must be exchanged with a bifunctional cap that attaches to the QD with one functionality and provides solubility and possible bioconjugation sites with the other. A wide variety of ligands can be used; each have their own advantages and disadvantages. For example, some limit dispersions of QDs to the basic pH range, whereas others increase the size considerably.[263,265]

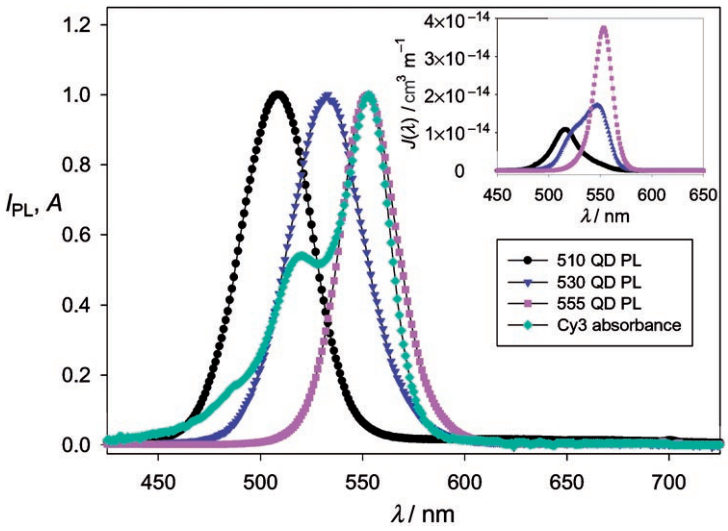


Figure 21. Normalized absorption spectrum of Cy3 and emission spectra of three QD solutions. The inset shows a plot of the resulting overlap functions $J(\lambda)$, which highlight the ability to tune the emission of the QD by changing its size to improve the spectral overlap with this acceptor.[271] Reproduced with permission from the American Chemical Society.

Mattoussi's group has been at the forefront of exploring QD FRET for bioassays. They have characterized and reported on D/A pairs of QD and dye-labeled proteins,[271,277] QD-based sensors for maltose and TNT,[4,278] surface-attached QD nanoassemblies,[279] QD-FRET-based reagentless biosensors,[280] the control of QD-donor FRET by a photochromic dye,[106] and the FRET-based structural elucidation of QD–protein bioconjugates (Figure 23).[281] These studies also demonstrate how FRET can be used with two different classes of fluorophores that differ in size by many orders of magnitude.

Other FRET applications with QDs include using DNA complementarity to attach gold quenchers to QDs[219] and using the quenching of these QDs to monitor avidin–biotin interactions.[221] The dynamics of DNA replication and telomerization have been monitored with QD donors that were conjugated to DNA primers and fluorescent nucleotide acceptors.[282] QDs have also been investigated as possible FRET donors in molecular beacons.[283] QDs located deep within lipid vesicles have been used as donors for

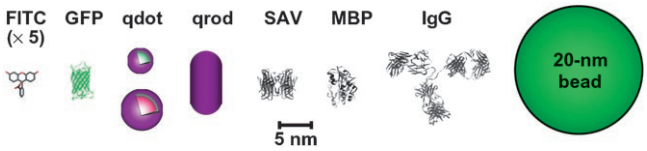


Figure 22. Comparison of the size of QDs and several comparable objects: FITC (fluorescein isothiocyanate), CdSe/ZnS QD (green: 4-nm diameter; red: 6.5-nm diameter), qrod (rod-shaped QD), SAV (streptavidin), IgG (immumoglobulin G). Figure generously provided by X. Michalet, UCLA, and reproduced with permission from reference [265].

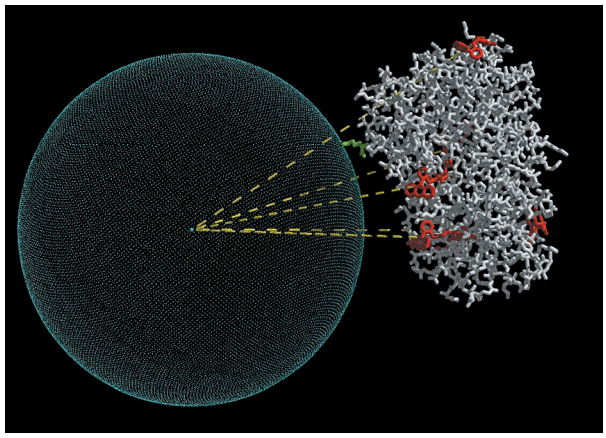


Figure 23. Side view of the structure of MBP as it self-assembles onto the surface of a QD. Six rhodamine red structures are highlighted in red. The distances from the center of the QD to each dye were determined by FRET (yellow). The crystallographic coordinates of the MBP were used in conjunction with these six distances to solve the structure of the MBP bioconjugate. Reproduced with permission from National Academy of Sciences USA.[281]

Diverse strategies also exist for attaching biomolecules to QDs, for example, covalent coupling,[261,262] electrostatic or metal-affinity-driven self-assembly, and biotin-avidin chemistry.[263,265,267,268,275,276] QDs consisting of various other binary and ternary semiconductor materials including ZnS, CdS, CdTe, PbSe, and CdHgTe with emissions ranging from the UV to the IR have also been synthesized (Figure 20a).[263–265]

The finite size of QDs presents an interesting predicament, since it can be both a benefit and a liability for FRET applications. The diameters of the CdSe/ZnS QDs shown in Figure 20b range from approximately 50 Å for the 510-nm QDs to more than 80 Å for the 610-nm QDs (not including the capping ligand, which can add between 20 and 100 Å to the overall size).[263,271,276,277] For many bioconjugates with a QD donor and a dye-labeled protein as acceptor, the R_0 value may actually fall within the radius of the QD, which results in a FRET efficiency that is relatively low for a D/A pair consisting of a single QD and a single acceptor.[4,271] However, it has been shown that by loading a central QD donor with multiple protein-based acceptors, the FRET efficiency can be increased in proportion with the cross section of the FRET acceptor.[263,271]

assaying interactions with other lipid-soluble and water-soluble dyes.[284] There is also a continuing discussion about using QDs as FRET-donating photosensitizers in photodynamic cancer therapy.[285,286]

There are far fewer examples of QDs as acceptors in biological contexts. There are two possible factors for this: 1) The broad absorbance profile, high extinction coefficients, and the large size of QDs cause QD to be excited as well as or better than any potential donor. 2) QDs have a longer lifetime ($\tau = 10$ to 50 ns) than typical fluorescent dyes ($\tau = 1$ to 5 ns).[277] Thus, the opportunity exists to use a different class of fluorophores, such as long-lifetime lanthanide chelates, as

donors in a FRET system, so as to exploit properties not found in "conventional" organic dyes. In view of the unique photophysical properties of QDs, we can expect their continued utilization in many FRET-based biological assays.

5. Multi-FRET Systems

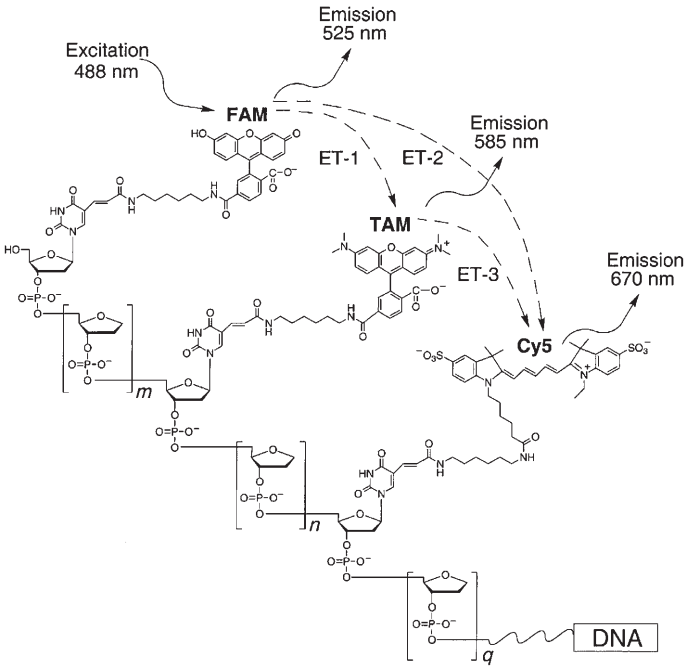
The naturally occurring multi-FRET biological systems are exemplified by the light-harvesting phycobilisomes.[287–291] These supramolecular complexes, found in blue-green cyanobacteria, red algae, and cryptomonad algae, function to extend the wavelength range for photosynthesis in the marine environment. Phycobilisomes consist of multiple phycobiliprotein subunits that can be pigmented or colorless; their composition varies widely depending on the light quality and the organism.[287–291]

An example of a multi-FRET function within a phycobilisome is the absorption of light by the phycobiliprotein R-phycoerythrin with subsequent energy transfer to C-phycocyanin and from there to allophycocyanin. The latter is connected through a linker chromophore to the photosystem II of the photosynthesis complex. Glazer and Stryer demonstrated that these tandem FRET probes could be adapted for sensitive cellular labeling and immunoassays.[292,293] Individually, these same fluorophores are also commercially available in the PBXL series.[123] The energy-transfer efficiency in this system approaches 100%—both the complexity and the efficiency of this naturally occurring energy-harvesting system are yet to be matched experimentally.

Biologically inspired synthetic multi-FRET systems have generally been used in two almost complementary configurations. In one case, defined biological structures are used to space or orient the fluorophores precisely.[5,47,294–296] In the converse case, multiple fluorophores are used to elucidate biological structures.[3,297] DNA is perhaps the most attractive biological platform for multi-FRET configurations for a number of reasons: 1) its predictable structure and chemistry; 2) the inherent ability to introduce fluorophores at specific sites;[5] 3) the ability to hybridize multiple dye-labeled oligonucleotides to a complementary strand;[298] 4) the ability to control the orientation of the attached fluorophores.[47]

DNA can be synthesized with multiple fluorophores or thiol, amine, biotin, and other modifications at specific terminal or internal sites. A change in the D/A spacing is facile in this configuration and allows fine tuning of FRET efficiency.[299,300] Such multilabeled DNA structures have been proposed as combinatorial fluorescence energy transfer (CFET) tags for information encoding. Tong et al. constructed eight CFET tags by altering the spacing between three fluorophores on a deoxyribose backbone to create different emission ratios of each color (Scheme 7).[295,296] In this configuration it is possible to excite at a single wavelength and use the different emission ratios as unique FRET signatures. The CFET tags have already been demonstrated in genotyping assays.

MBP has also been used to test different protein-based multi-FRET configurations.[26,52] Hellinga used orthogonal



Scheme 7. Structure of a CFET tag, which is constructed through attachment of chromophores to the modified thymidine residues of a nucleotide backbone. The FAM donor is excited at 488 nm and transfers energy to the proximal TAM and the terminal Cy5. TAM acts as a relay to forward energy to the Cy5. The spacing between FAM and TAM is controlled by the number of sugar phosphates *m*, and *n* defines the spacing between TAM and Cy5. Through changing the spacings, the emission can be tuned so that a unique ratiometric signature is produced. The CFET tag can be attached to DNA at point *q*, whose spacing can also be controlled. Figure courtesy of A. Tong.[295,296]

protein labeling to create a triply labeled MBP: the labels, FAM, tetramethylrhodamine (TMR), and Cy5, form a FRET relay that responds to maltose according to the change in the FRET ratio between FAM and Cy5, while the central TMR acts as a relay.[26] In another approach, a Cy3–MBP conjugate was used as a relay between a QD and a Cy3.5-labeled analogue of maltose, bound in the central binding pocket of the MBP (Figure 24). Although maltose sensing in this QD–MBP displacement sensor is based on changes in the ratio of FRET emission from MBP–Cy3 and Cy3.5, the sensor is "driven" by the QD, which is the primarily excited participant (Figure 25). This approach was helpful in overcoming inherent limitations of the D/A distance.[52] A multi-FRET format with 148 donors and 24 acceptors has been used to elucidate the structure of tarantula hemocyanin.[301] A FRET system with three fluorophores for a high-throughput drug screening format has also been reported.[302] Recently, Wang and Tan incorporated a combination of three organic dyes into silicon nanoparticles and varied the ratio of these tandem dyes to tune the FRET-mediated emission signatures.[303] This strategy represents an interesting functional hybrid that combines elements of silicon NPs,[237–240] CU dots,[246] and TransFluospheres.[56]

Multi-FRET systems have tremendous potential for elucidating protein structures and interactions, and a worth-

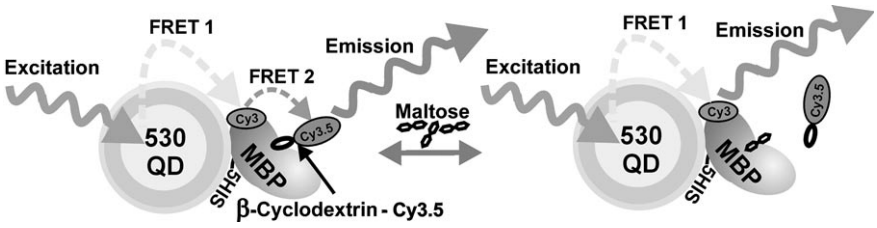


Figure 24. Schematic function of a multi-FRET QD maltose sensor. A 530-nm QD is surrounded by about 10 MBPs (only one shown), each labeled with a single Cy3 molecule (absorption maximum 556 nm, emission maximum 570 nm). β-Cyclodextrin, an analogue of the primary maltose analyte, is labeled with Cy3.5 (β-CD-Cy3.5, absorption maximum 575 nm, emission maximum 595 nm); it binds specifically in the binding pocket of MBP to complete the sensor complex. Excitation of the QD results in excitation of the MBP-Cy3 (FRET 1), which in turn excites the β-CD-Cy3.5 (FRET 2). Added maltose displaces β-CD-Cy3.5 and leads to increased emission of Cy3.

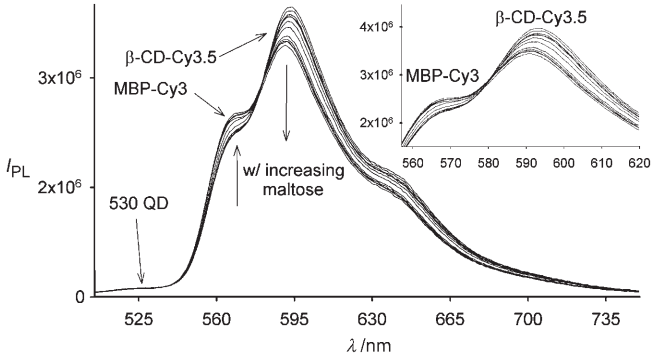


Figure 25. Maltose sensing using the configuration shown in Figure 24. Inset: Close up of the MBP-Cy3 and β-CD-Cy3.5 fluorescence portions. Reproduced with permission of the Nature Publishing Group.[4]

while strategy for their construction is in particular the combination of different types of functionalized fluorophores.

6. New Materials

With the growth in recent years of our understanding of FRET and its applications, possibilities have arisen for moving beyond the distance limitations of traditional FRET applications ($R_0 = 60$–90 Å, $r = 100$–120 Å). For example, Jares-Erijman and Jovin[13] point out that certain FRET parameters, including R_0, may be more plastic depending upon experimental configuration than previously thought. Specifically, the distance dependence of energy-transfer from a point to a plane could vary with the fourth power of separation rather than the sixth.[13] Strouse and co-workers investigated this aspect by using variable-length DNA that was labeled at one end with FAM and at the other with a 1.4-nm Au cluster (Figure 26).[304] The Au cluster acts as a dipole surface and demonstrates $1/R^4$ distance dependence of the surface energy transfer (SET). An SET radius d_0 (analogous to R_0) can be extrapolated.[304] SET may provide a distance resolution of up to 220 Å or more, which is twice the distance resolution measurable with traditional dye-based FRET pairs.

In a related approach, which is admittedly not directly based on energy transfer, Alivisatos and co-workers demonstrated a "molecular ruler" based on the plasmon coupling of single Au or Ag nanoparticles.[305] Plasmon coupling allows single pairs of nanoparticles separated by distances up to 700 Å to be monitored. However, deducing absolute distance values is complicated by factors such as the refractive index and light scattering. Interestingly, these two processes specifically necessitate the use of fluorophores distinct from traditional organic dyes.

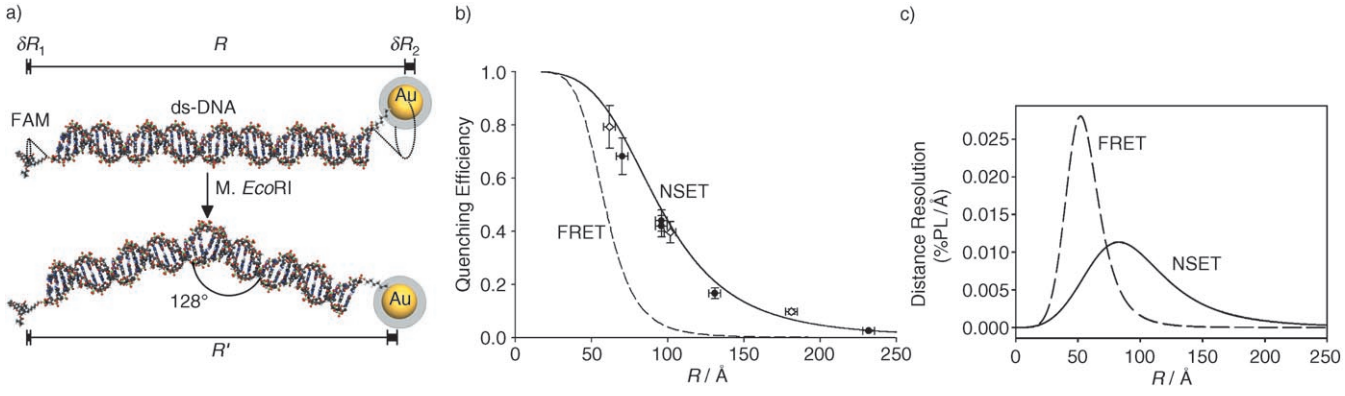


Figure 26. a) Schematic of the FAM-DNA-Au system: FAM is appended to DNA with a 1.4-nm Au particle attached at the other end. Flexible C_6 linkers produce cones of uncertainty (δR) for both appended moieties. Binding of M. *Eco*RI methyltransferase bends the DNA by 128°, which alters distance R to R' and results in a new D/A distance. b) Energy-transfer efficiency plotted against distance of separation R between FAM and Au. Filled circles represent DNA lengths of 15 bp, 20 bp, 30 bp, and 60 bp. Efficiencies after M. *Eco*RI binding are shown in open symbols. The dashed line is the theoretical FRET efficiency and the solid line is the theoretical SET efficiency. c) Distance-dependent length resolution of FRET and SET mechanisms. The intersection of the curves is the distance at which the two methods have identical resolution. Figure generously provided by G. Strouse F.S.U. and reproduced from reference [304] with permission from the American Chemical Society.

7. Summary and Outlook

Despite the numerous examples of FRET systems with divergent materials presented in this Review, FRET remains an underused and underappreciated analytical tool. Possible applications are numerous; for example, most commercial DNA sequencers utilize energy-transfer primers or terminators for fluorescent labeling as this simplifies the instrumental optical systems needed yet few realize this realiance.[16,306–308] There are few other bioanalytical techniques that can in so many different experimental formats consistently provide accurate intramolecular distance measurements in the nanometer range. We are fortunate to be part of an era in which not only is sensitive detection equipment available, but there are also many disparate materials that can be used as donors and acceptors in FRET systems, and numerous methods to label biomolecules. In particular, the use of nontraditional combinations with materials other than the usual organic donor and acceptor dyes will expand the applicability of FRET analysis. We predict six areas that will benefit the most in the near future: 1) studies of protein and peptide folding kinetics; 2) the elucidation of macromolecular interactions; 3) multicolor analysis, especially in vivo; 4) clinical and in vitro assays; 5) novel nanomaterials; 6) single-molecule FRET analyses.[310,311]

Addendum

During the production of this Review, several pertinent papers were published which deserve mention. In a fascinating example of FRET between disparate classes of materials, So et al. used BRET to illuminate QDs in the absence of external excitation and demonstrated this for in vivo deep tissue imaging.[312] A multi-FRET construct consisting of five perylene bisimide dyes on a calixarene backbone was demonstrated for potential use in light-harvesting arrays.[313] Simultaneous multiplex FRET with up to four QD donors was demonstrated,[314] and a very informative review article on BRET applications for determining protein–protein interactions was published.[315]

Abbreviations

BFP	blue fluorescent protein
BL	bioluminescence
BRET	bioluminescent resonance energy transfer
CFET	combinatorial fluorescence energy transfer
CFP	cyan fluorescent protein
CL	chemiluminescence
CRET	chemiluminescent resonance energy transfer
D/A	donor/acceptor
FAM	fluorescein
FRET	Förster or fluorescence resonance energy transfer
FP	fluorescent protein
GFP	green fluorescent protein
HRP	horeradish peroxidase
MBP	maltose-binding protein
NP	nanoparticle
pcFRET	photochromic FRET
QD	quantum dot
QY	quantum yield
R_0	Förster distance
SNP	single nucleotide polymorphism
SET	surface energy transfer
τ	excited-state fluorescent lifetime
YFP	yellow fluorescent protein

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